METHOD AND KIT FOR PRIMER BASED MULTIPLEX AMPLIFICATION OF NUCLEIC ACIDS

BACKGROUND

One important issue for the effective containment and control of any agent is a timely and accurate diagnosis of patients infected with that agent. One aspect of such a diagnosis includes differential diagnosis. By differential diagnosis it is meant the determination and identification of those patients having a disease state or condition caused by a disease agent of interest, and those patients having a disease state condition caused by one or more secondary agents that result in a similar clinical manifestation. This differential diagnosis is critical since the symptoms/clinical manifestations observed in conditions caused by the disease agent of interest may also be observed in conditions caused by the secondary agents. Therefore, the possibility of misdiagnosis is a significant issue. Such misdiagnosis can result in both false positives, and false negatives. Each of these types of misdiagnosis has a detrimental impact of the containment and control of the disease agent. For example, a false negative will allow an infected individual to continue to spread the disease agent to the general population. A false positive will result in an increased burden on the healthcare system and on the individuals required to undergo needless treatment.

False positives and false negatives can be of special concern in cases of bioterrorism, where accurate and rapid identification of the causative agent is required for containment, control and an effective public health response. The growing concern regarding the use of bioterrorism has prompted Federal health agencies to accelerate measures to protect the public from such attacks. In February, 2002, the National Institute of Allergy and Infectious Diseases (NIAID) released its Biodefense Research Agenda for CDC category A, B and C agents. One of the goals of this research agenda was the development of diagnostic test applicable to agents that may be used in bioterrorism. The Agenda stated "A successful response to a bioterrorist threat requires diagnostics that can identify the pathogen involved. However, the initial clinical signs and symptoms of many agents considered biothreats are nonspecific and resemble those of common infections. The ability to rapidly identify the introduction of a bioterrorism organism or toxin will require diagnostic tools that are highly sensitive, specific, inexpensive, easy to use, and located in primary care settings."

As an example of the problems and issues discussed above, consider the recent outbreak of the severe acute respiratory syndrome (SARS). The clinical symptoms of SARS, especially in the early stages, included fever, chills and moderate to severe coughing. Obviously, these symptoms are observed in a number of conditions caused by other agents. Other agents capable

of causing conditions with SARS-like symptoms include, but are not limited to, respiratory syncytial virus, parainfluenzaviruses type 1 and type 3, influenza A and B viruses, enterovirus, adenovirus, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*.

There are three classes of diagnostic tests commonly used in the detection of disease agents: i) ELISA tests; ii) cell culture methods; and iii) molecular tests. Each of these tests has their own advantages and disadvantages. The ELISA (Enzyme Linked Immunoabsorbant Assay) is an antibody test. It detects antibodies to the disease agent in the serum of patients reliably by day 21 after the onset of clinical symptoms. ELISA is specific, but the detection comes too late for detection to be useful in disease management. It can not provide the much needed early information required for the containment and control of the disease agent.

Cell culture methods detect the presence of live agent. A sample is taken from an individual suspected of being infected with the disease agent and the disease agent is propagated in cultured cells or cultured according to defined conditions on selective culture media. Either process is a time-consuming, demanding and dangerous task, but it is the only means to show the existence of the live agent. As with the ELISA, the test is relatively specific, but the detection comes too late for detection to be useful in disease management.

The molecular tests generally use any one of a number of variations on the polymerase chain reaction (PCR). PCR can detect genetic material of an agent in various specimens (blood, stool, respiratory secretions or body tissue) from the individual suspected of being infected by the disease agent. Existing PCR tests are very specific, but lack sensitivity. This is because the agent may not yet be present in the patient specimens or the amplification and detection schemes fail to identify the genetic material. Therefore, a negative test can't rule out the presence of the disease agent in an individual.

Multiplex PCR allows the amplification of target sequences from multiple organisms in one reaction using multiple sets of locus specific primers. Therefore, multiplex PCR is suited to differential diagnosis. However, multiplex PCR methods have limitations. There are two major problems associated with the multiplex PCR method. One is that each target sequence (or locus) to be amplified has its own amplification efficiency. The locus specific amplification efficiency is determined by multiple factors including the composition of the primer targets, binding affinity of the primers to their targets, priming efficiency of the primers and availability of reaction components. Combining multiple target loci in one reaction may introduce incompatibility between various primer sets which results in preferential amplification or inhibition of some amplification reactions. The second issue is the identification of the optimal primer to locus ratio. If the primer concentrations are set too high, primer dimmers and background amplification will occur. If, however, the primer concentrations are too low, the desired exponential amplification of

the target sequence will not occur.

In order to optimize multiplex PCR, the concentrations of primers, buffer, dNTPs, enzyme, and $MgCl_2$ need to be determined empirically for each set of primer combinations. It is a time consuming process which needs to be conducted for each lot of the produced assay. A successful multiplex PCR is not guaranteed even after exhaustive optimization experiments.

The present disclosure provides a quick, accurate molecular diagnostic method for the diagnosis of a disease agent and/or the differential diagnosis of a disease agents in the presence of one or more secondary disease agents. Briefly nucleic acid samples are obtained from samples suspected of containing the disease agent and/or secondary disease agents; the nucleic acid may be DNA or RNA (either positive strand or negative strand) or a combination thereof. A multiplex amplification reaction is used to amplify pre-determined target sequences from the nucleic acid through one amplification reaction in one vial. The amplification products containing the target sequences are detected and differentiated using a multiplex detection strategy. The detection of the target sequence from a disease agent or secondary disease agent indicates its presence in the sample. Using the method disclosed herein, a diagnosis or differential diagnosis of a disease agent can be made in as little as 3 hours. The high throughput ability allows the analysis of hundreds of samples per day without the need for complex and time consuming optimization procedures for each primer combination. Such methods are lacking in the art.

BREIF DESCRIPTION OF THE DRAWINGS

FIG.S. 1A and 1B are illustrations of two embodiment of the Tem-PCR amplification process.

FIG. 2 is an illustration of one embodiment of the direct detection methodology.

FIG. 3 is an illustration of one embodiment of the indirect detection methodology.

DETAILED DESCRIPTION

The method described herein, termed the Multiplex Analysis System (MAS) provides a rapid and convenient format for diagnosis of disease agents and/or differential diagnosis of disease agents and secondary disease agents. As used in this specification, an "agent" means any organism, regardless of form, that incorporates a nucleic acid and that causes or contributes to an infection, a symptom, or a condition, including, but not limited to a bacteria (list classes), a virus (the virus may have a DNA genome, a negative strand RNA genome or a positive strand RNA genome) or a parasite. The infection, symptom, or condition caused by or related to the disease agent sought to be diagnosed is referred to in this specification as the "disease state". As used in this specification, a "disease agent" means any agent that causes or contributes to disease state sought to be diagnosed. In one embodiment, the disease agent and/or the disease state sought to

be diagnosed will be determined in advance by a healthcare provider or other person. In one embodiment the disease agent may be involved in bio-weapons programs, such as the organism described as potential biothreats which are described in the NIAID Biodefense Research Agenda. As discussed herein, the MAS can be used in differential diagnosis. When discussing differential diagnosis, reference will be made to the disease agent and one or more secondary disease agents. As used in this specification, the "secondary disease agent(s)" means any agent that presents a similar clinical presentation to the disease state caused or contributed to be the disease agent. As a result, a differential diagnosis using the MAS will be able to accurately determine the presence of the disease agent if the disease agent is present, as well as the presence of any secondary disease agents that may be present.

As used interchangeably in this disclosure, the terms "nucleic acid molecule(s)", "oligonucleotide(s)", and "polynucleotide(s)" include RNA or DNA (either single or double stranded, coding, complementary or antisense), or RNA/DNA hybrid sequences of more than one nucleotide in either single chain or duplex form (although each of the above species may be particularly specified). The term "nucleotide" is used herein as an adjective to describe molecules comprising RNA, DNA, or RNA/DNA hybrid sequences of any length in single-stranded or duplex form. More precisely, the expression "nucleotide sequence" encompasses the nucleic material itself and is thus not restricted to the sequence information (i.e. the succession of letters chosen among the four base letters) that biochemically characterizes a specific DNA or RNA molecule. The term "nucleotide" is also used herein as a noun to refer to individual nucleotides or varieties of nucleotides, meaning a molecule, or individual unit in a larger nucleic acid molecule, comprising a purine or pyrimidine, a ribose or deoxyribose sugar moiety, and a phosphate group, or phosphodiester linkage in the case of nucleotides within an oligonucleotide or polynucleotide. The term "nucleotide" is also used herein to encompass "modified nucleotides" which comprise at least one modification such as (a) an alternative linking group, (b) an analogous form of purine, (c) an analogous form of pyrimidine, or (d) an analogous sugar. The polynucleotide sequences of the invention may be prepared by any known method, including synthetic, recombinant, ex vivo generation, or a combination thereof, as well as utilizing any purification methods known in the art.

The MAS described herein is capable of detecting disease agents that cause or contribute to a variety of disease states. The MAS can be used in differential diagnosis to determine if a specific disease agent is present and to determine if secondary disease agents are present. However, it is not required that the MAS be used in a differential diagnosis application. The MAS can also be used to diagnose the presence or absence of genetic mutations related to disease states, the presence or absence of single nucleotide polymorphisms (SNPs), to determine gene

expression profiling, and to determine gene dosage mutations. Applications of these alternative uses of the MAS are described in co-pending U.S. application serial number 10/284,656. Other uses of the MAS will be appreciated by those skilled in the art.

The MAS described may comprise three steps: i) nucleic acid isolation; ii) multiplex amplification to amplify the nucleic acid sequences from the disease agent or secondary disease agent containing the target sequence(s); and iii) multiplexed detection of the target sequences amplified in step (ii). In the embodiment of the MAS described the simultaneous isolation of both RNA (both positive and negative strand) and DNA is provided. Simultaneous isolation of both RNA and DNA is advantageous since the disease agent and the secondary agents may have different nucleic acid genomes. Without dual amplification of DNA and RNA, multiple samples would be required to be tested. In an alternate embodiment, where the disease agent and the secondary disease agents each have an identical type of nucleic acid genome, the nucleic acid isolation step may isolate only one type of nucleic acid. The MAS is highly adaptable, allowing new agents and mutants of known and new agents to be included when they become known through appropriate design of the primer sequences used in the multiplex amplification step. As will be discussed in more detail below, in one embodiment, the multiplex amplification strategy used in the MAS utilizes unique and heretofore unappreciated methods to reduce incompatibility between various primer pairs and to increase sensitivity and specificity of the multiplex amplification reaction over those of currently available methods. The entire MAS procedure can be accomplished in as little as three hours.

The MAS is described generally so that its application can be understood. The MAS method can be used to determine the presence of a disease agent from a sample obtained from an individual suspecting of harboring said disease agent, and to therefore, identify and diagnose those individuals who have a disease state caused by or contributed to by the disease agent. The MAS may be used in differential diagnosis involving the disease agent and one or more secondary disease agents. Although the MAS can be used to determine the presence of any disease agent, an example is provided illustrating the differential diagnosis where SARS is the disease agent, and where respiratory syncytial virus A and B, HPIV 1 and 3, influenza A and B, enterovirus, adenovirus 4 and 21, *C. pneumoniae* and *M. pneumoniae* are the secondary disease agents. However, the MAS can be used to determine the presence of any disease agent and secondary disease agent through appropriate design of the multiplex amplification.

The various components of the MAS are described in greater detail below. It should be appreciated that while certain embodiments are discussed in regard to these components, other methods known in the art for accomplishing the same ends should be considered within the scope of the present disclosure. In addition, various embodiments of the MAS may use different

methods of carrying out the steps described below, depending on the purpose of the MAS, the nature of the disease state, and the nature of the disease agent and one or more secondary disease agents.

Nucleic Acid Isolation

In one embodiment of the MAS, a nucleic acid isolation step is used that isolates both RNA and DNA in one reaction. In an alternate embodiment, RNA and DNA may be isolated independently and then combined for use in the MAS. In yet another alternate embodiment, when only one type of nucleic acid is required to be isolated (such as when all the disease agents and secondary disease agents of interest have the same type of nucleic acid genome), nucleic acid isolation methods that isolate only RNA or DNA may be used. A variety of techniques and protocols are known in the art for simultaneous RNA and DNA isolation and the separate isolation of each and such techniques and protocols may be used. The nucleic acid isolation described may be used to isolate nucleic acid from a variety of patient samples or sources. The types of patient samples/sources include, but are not limited to, nasal/pharyngeal swabs, saliva, sputum, serum, whole blood and stool.

The nucleic acid isolation method may satisfy one or more of the following requirements. First, the nucleic acid isolation method may inactivate any disease agent and any secondary disease agents that may be present in the patient samples. As a result, the risk to laboratory and healthcare personnel is reduced. Furthermore, the remaining steps of the MAS can be completed without the requirement for stringent bio-containment procedures if desired. In addition, the method may allow for the removal or PCR and RT-PCR inhibitors and other unwanted compounds from the isolated nucleic acid.

In one embodiment, a dual RNA/DNA isolation method is used employing a trizol based reagent for initial isolation of RNA and DNA from patient samples. Upon contact with patient samples, the phenol and high salt reagents in the trizol effectively inactivate any disease agent or secondary disease agent that may be present in the patient sample. In order to allow for the dual isolation of RNA and DNA in the same phase with a single step, the pH of the trizol solution may be adjusted towards neutral (instead of acidic). After the RNA and DNA are isolated from the patient samples, a silica based column may be used to further isolate the RNA and DNA. The use of silica based columns allows for wash steps to be performed quickly and efficiently while minimizing the possibility of contamination. The wash steps may be used to remove PCR and RT-PCR inhibitors. The column method for nucleic acid purification is advantageous as it can be used with different types of patient samples and the spin and wash steps effectively remove PCR or RT-PCR inhibitors.

In one embodiment, the nucleic isolation is carried out using the dual RNA/DNA isolation

kit provided by Omega Bio-Tek according to manufacturer's instructions. Briefly, 250µl of subject sample is added to 1 ml of nucleic acid isolation reagent (trizol reagent) followed by 250µl chloroform. The mixture is thoroughly mixed, such as by vortexing. Samples are centrifuged at 12,000 x g for 10 minutes to separate the aqueous and organic phases. The upper aqueous phase is carefully transferred into a new 1.5 ml centrifuge tube. An equal volume of 70% ethanol is added and the samples mixed, such as by vortexing. The sample are applied onto a HiBind spin column set in a collection tube and centrifuged at 10,000 x g for 15 seconds. The flow through is discarded. 500µl of Wash Buffer I is added to the column and the column centrifuged for 15 seconds. The column is washed with 500µl of Wash Buffer II, twice, for 15 seconds each. The RNA/DNA was eluted by adding 50µl of RNase-free water and centrifuging at maximum speed for 1 minute. The nucleic acid samples may then be used for the amplification steps as described below.

In addition to the trizol method described above, other methods may be used to isolate RNA and/or DNA. In an alternate embodiment, LNA-conjugated magnetic beads are used. LNA (Locked Nucleic Acids) are a class of nucleic acids containing altered nucleosides whose major distinguishing characteristic is the presence of a methylene bridge between the 2'-O and 4'C atoms of the ribose ring. LNA nucleosides containing the five common nucleobases that appear in DNA and RNA (A,T,U,C,G) can base-pair with their complementary nucleosides according to Watson-Crick rules. The molecular differences between normal nucleosides and LNAs give rise to differences in the stability of nucleic acid duplexes formed between LNA containing nucleic acids and non-LNA containing nucleic acids. Typically, each LNA nucleotide incorporated increases the T_m of a LNA/DNA nucleotide complex by 2-6°C as compared to a corresponding DNA/DNA complex. LNA-containing oligonucleotides capable of binding to the nucleic acid of disease agent and secondary disease agents (either through specific or non-specific interactions) could be linked to magnetic beads in order to isolate said nucleic acid. The LNA-containing oligonucleotides will bind to nucleic acid of the disease agent or secondary disease agent; separation may then be achieved by a simple magnetic separation and washing step. The process also allows the removal of PCR inhibitors. The LNA-containing oligonucleotide/magnetic bead conjugates could be mixed directly with PCR or RT-PCR reagents for use in the multiplex amplification step. Using the LNA-containing oligonucleotide magnetic beads, patient samples with high volumes can be efficiently processed. The LNA method may be used conjunction with the triazol dual extraction method described above or any other method known in the art or described in this specification for isolating DNA and/or RNA.

Multiplex Amplification

A variety of multiplex amplification strategies may be used with the MAS. Many such amplification strategies are known in the art. The multiplex amplification strategy may use PCR, RT-PCR or a combination thereof depending on the type of nucleic acid contained in the disease agent and secondary disease agent. For example, if an RNA genome is present, RT-PCR may be utilized. The methodology of PCR and RT-PCR are well known in the art. In one embodiment, the multiplex amplification strategy employed in the MAS is unique. This unique multiplex amplification strategy is termed target enriched multiplex PCR (Tem-PCR) and allows the efficient amplification of one or more target sequences from a disease agent and/or secondary disease agents without extensive empirical testing of primer combinations and amplification conditions as is required with other multiplex amplification methods known in the art. As used in this specification, a "target sequence" is a nucleotide sequence from a disease agent or a secondary disease agent that is to be amplified and ultimately detected; the target sequence is contained in a larger nucleic acid sequence amplified by the primer sets described herein. Each target sequence for amplification is selected so that on detection (as discussed below) it allows the identification of a disease agent or secondary disease agent, if either is present in the sample. The detection of the amplified target sequence (either directly or indirectly- see discussion below) will indicate the presence and identity of the disease agent or the secondary disease agent and thereby diagnose the relevant disease state. In one embodiment, a single target sequence is selected for amplification from each disease agent and each secondary disease agent to be tested. In an alternate embodiment, more than one target sequence is selected for amplification from each disease agent and each secondary disease agent. In yet an additional embodiment, more than one target sequence is selected for amplification from the disease agent and a single target sequence is selected for amplification from each secondary disease agent.

The principle of Tem-PCR is illustrated in FIGS. 1A and 1B. FIG. 1A illustrates the use of 3 primer oligonucleotide pairs for the amplification of the target sequence. The primer oligonucleotides are indicated as F_{out} (outside forward primer) F_{in} (inside forward primer), R_{out} (outside reverse primer), R_{in} (inside reverse primer), FSP (forward super primer) and RSP (reverse super primer). The 3 primer pairs illustrated in FIG. 1 comprise 2 pairs of target enrichment primers (F_{out} and R_{out}; and F_{in}, and R_{in}) and 1 pair of target amplification primers (FSP and RSP). As discussed below, additional target enrichment primers and target amplification primers may be incorporated as desired. The target enrichment primers, in this embodiment, F_{out} and R_{out} and F_{in}, and R_{in}, are designed to hybridize specifically to the nucleic acid containing the target sequence and to bracket the target sequence as illustrated in FIG. 1A. Therefore, the first set of target enrichment primers, F_{out} and R_{out}, binds the nucleic acid containing the target sequence and brackets the target sequence (one of F_{out} and R_{out} is on the 5',

or left, side of the target sequence and the other is on the 3', or right, side of the target sequence). The second set of target enrichment primers, F_{in}, and R_{in}, binds the nucleic acid containing the target sequence and brackets the target sequence as described for the first set of target enrichment primers. The second set of target enrichment primers binds to the inside of the first set of target enrichment primers as shown in FIG. 1A. This location is defined as proximate to the target sequence; in other words, the second set of target enrichment primers binds the nucleic acid containing the target sequence such that the second set of target enrichment primers are located closer to the target sequence as compared to the first set of target enrichment primers.

Each of the primers in at least one of the first or second primer enrichment pairs further comprises a super primer binding tag on its 5' end. The super primer binding tag is an oligonucleotide sequence that is identical to the sequence of the target amplification primers (RSP and FSP). During amplification during the TemPCR process, the primer pair containing the super primer binding tag is copied into its complement to create a binding site for the at least one pair of target amplification primers (FSP and RSP) to allow exponential amplification of the target sequence. In the embodiment shown in FIG. 1A, the second target enrichment primer pair comprises the super primer binding tag, with F_i containing a super primer binding tag identical to the sequence of the FSP and R_i containing a super primer binding tag identical to the sequence of the RSP. The outside primers may comprise the super primer oligonucleotide tag is desired, with the principles of operation being the same as described above.

The specificity of the hybridization between the target enrichment primers and their nucleic acid sequences can be adjusted by increasing or decreasing the length of the primer sequence responsible for hybridization as is known in the art. In general, a shorter primer sequence will give increased specificity while longer primer sequence will provide greater hybridization efficiency. Furthermore, increasing or decreasing the lengths of the primer sequence responsible for hybridization may also determine which primers are active during the various stages of the TemPCR amplifications process (see discussion below). In one embodiment, the length of the target enrichment primers is from 10 to 50 nucleotides. In an alternate embodiment, the length of the target enrichment primers is from 10 to 40 nucleotides. In yet another alternate embodiment, the length of the target enrichment primers is from 10 to 20 nucleotides. The primers in each set of the target enrichment primers may be different lengths if desired. For example, in one embodiment, the target enrichment primers F_{out} and R_{out} are 15-25 nucleotides in length and the target enrichment primers F_{in} and R_{in} are 35 to 45 nucleotides in length (with such length not including the super primer binding tag).

The first set of target amplification primers, FSP and RSP in FIGS. 1A and 1B, are common primer sequences and are used for the universal amplification of nucleic acid amplified

during the target enrichment step (which contains the target nucleic acid). In one embodiment, the length of the target amplification primers is from 10 to 50 nucleotides. In an alternate embodiment, the length of the target amplification primers is from 10 to 40 nucleotides. In yet another alternate embodiment, the length of the target amplification primers is from 10 to 20 nucleotides.

The target enrichment primers are used at low concentrations for enrichment (i.e. limited amplification) of the target sequence, while the target amplification primers are used at high concentration for exponential amplification of the target sequences. Since any convenient target sequence can be chose for amplification and detection, the sequence of the nested primers are dictated only by the nature of the nucleic acid sequence flanking the target sequence. Therefore, the target enrichment primers can be designed with minimal constraint on their composition. The use of at least one set of nested primers that does not contain the oligonucleotide tag (such as F_{out} and R_{out} in FIG. 1A) may increases the efficiency of the initial reverse transcription reaction. Multiple sets of target enrichment primers may enhance the sensitivity and specificity of the assay by allowing more opportunity and combinations for the nested primers to work together to provide target sequence enrichment. In addition, since the target enrichment primers are present at low concentrations, are not labeled, and are not responsible for the exponential amplification of the target sequences, the design of the target enrichment primers does not raise significant issues regarding compatibility regarding the various combinations of target enrichment primers. As discussed below, the exponential amplification is carried out by the super primers. This aspect of Tem-PCR allows the simple and rapid modification of the assay to detect additional disease agents and secondary disease agents.

Although the embodiments illustrated in FIGS. 1A and 1B show the use of one or two sets of target enrichment primers to amplify a given nucleic acid containing the target sequence, more than 2 sets of target enrichment primers may be used if desired. In one embodiment, 3 to 6 sets of target enrichment primers are used in the Tem-PCR reaction. In an additional embodiment, 3 to 5 sets of target enrichment primers are used. In an alternate embodiment, 3 to 4 sets of target enrichment primers are used.

More than 1 set of target amplification primers may also be used. When more than 1 set of target amplification primers are used, the sequences of the multiple sets of target amplification primers are selected so that they are compatible with one another in the exponential amplification step. In other words, the multiple sets of super primers would share similar T_ms when biding to the super primer binding sites on the amplified target nucleic acid and have similar amplification efficiencies. Multiple target amplification primers may be used when one or more of the disease agent or secondary disease agents are present at different titers/concentrations. If there is a

significant difference in titer, then with only 1 set of target amplification primers, preferential amplification of the high titer agent may occur. This could result in a false negative diagnosis for the agent present at the lower titer. Such biased amplification may be avoided by using multiple sets of target amplification primers. In one embodiment, 2-8 sets of target amplification primers are used. In an alternate embodiment, 2-6 sets of target amplification primers are used. In yet another alternate embodiment, 2-4 sets of target amplification primers are used.

The target amplification primers are used at high concentrations. The sequence of the target amplification primers are the same for each target sequence to be amplified if one set of target amplification primers are used, or the target amplification primers are designed to share have similar amplification characteristics for each target sequence to be amplified if multiple sets of target amplification primers are used. In one embodiment, both of the target amplification primers incorporate a means for detection that enables the amplified products to be detected and/or manipulated as described below. In an alternate embodiment, only 1 of the two target amplification primers incorporates a means for detection. In yet another alternate embodiment, only the RSP of the target amplification primers incorporates a means for detection. As used in this specification, a means for detection may be any element that is known in the art, such as a chemical element, an enzymatic element, a fluorescent element, or a radiolabel element. In one embodiment, the means for detection may be a fluorescent element, such as, but not limited to, a Cy-3 label. The fluorescent element may be directly conjugated to the super primer sequences or may be indirectly conjugated. In the case of indirect conjugation, the means for detection may be a biotin molecule (i.e. a chemical element) and the fluorescent element may be conjugated to an avidin or streptavidin molecule. The detection means may be manipulated as described below.

The target enrichment primers used in the Tem-PCR method are not used in a two-step PCR reaction as is commonly known in the art for nested primer applications. In Tem-PCR the target enrichment primers are used in a one step PCR reaction. Since the target enrichment primers are present in low concentrations, the target enrichment serve a target-enrichment purpose, rather than a target amplification purpose. The concentration of the target enrichment primers is not sufficient for exponential amplification of the target sequences. The target enrichment primers also serve to open up local template structures and therefore increase amplification sensitivity and/or efficiency. The exponential amplification of the sense target sequence is accomplished using the FSP and RSP.

As used in this specification, a "low concentration" when used to described the concentration of the target enrichment primers means a concentration of primers that is not sufficient for exponential amplification of the given target sequence(s) but is sufficient for target enrichment of the given target sequences. This low concentration may vary depending on the

nucleotide sequence of the nucleic acid containing the target sequence to be amplified. In one embodiment, a concentration of target enrichment primers is in the range of 0.002 μM to less than 0.2 μM. In another embodiment, a concentration of target enrichment primers is in the range of 0.002 μM to 0.15 μM. In an alternate embodiment, a concentration of target enrichment primers is in the range of 0.002 μM to 0.1 μM. In yet another alternate embodiment, a concentration of target enrichment primers is in the range of 0.002 μM to 0.05 μM. Other concentration ranges outside those described above may be used if the nature of the nucleic acid sequence containing the target sequence to be amplified is such that concentrations of target enrichment primers below or above the ranges specified are required for target enrichment without exponential amplification. The various target enrichment primers may be used in different concentrations (i.e. ratios) or at the same concentration.

As used in this specification, a "high concentration" when used to described the concentration of the target amplification primers (FSP and RSP) means a concentration of primers that is sufficient for exponential amplification of the given target sequence. In one embodiment, a concentration of target amplification is in the range of 0.2 μM to 2.0 μM. In another embodiment, a concentration of target amplification primers is in the range of 0.2 μM to 1.0 μM. In an alternate embodiment, a concentration of target amplification primers is in the range of 0.2 μM to 0.8 μM. In yet another alternate embodiment, a concentration of target amplification primers is in the range of 0.2 μM to 0.4 μM. Other concentration ranges outside those described above may be used if the nature of the nucleic acid sequence containing the target sequence to be amplified is such that concentrations of target amplification primers below or above the ranges specified are required for exponential amplification. The super primers may be used in different concentrations (i.e. ratios) or at the same concentration.

As a general rule, a primer concentration in the range of 0.2 μM is generally used as a starting point for primer concentrations in order to achieve exponential amplification of a given target sequence. The target enrichment primers and the target amplification primers may be used in various ratios to each one another as discussed herein.

During the amplification process when two sets of target enrichment primers are used (FIG. 1A), the two sets of target enrichment primers will generate four possible amplified products, each containing the target sequence: F_{out}/R_{out}; F_{out}/R_{in}; F_{in}/R_{out}; and F_{in}/R_{in}. The super primer binding tag will be incorporated into any amplification product amplified using a target enrichment primer comprising the super primer binding tag, such as the F_{in} or R_{in} primer in FIG. 1A. However, the super primer binding tag is not useful for exponential amplification by the target amplification primers at this point since the super primer binding tag is identical to the sequence of the target amplification primers. In order to generate a super primer binding site, the

amplification products containing the super primer binding tag must be amplified a second time in the opposite direction of the first amplification. The multiplex amplification protocol (discussed below) utilizes a multi-step procedure that allows for incorporation of the super primer binding tag into the amplification products. These steps are referred to as the target enrichment steps and the selective amplification steps. The target enrichment step may be used on its own or in combination with the selective amplification step. The target enrichment and selective amplification steps are optimized to provide conditions required for the low concentration target enrichment primers to hybridize to their hybridization targets for target enrichment. These steps create sufficient amplified product to serve as the basis for the exponential amplification procedure to be carried out by the target amplification primers. Therefore, at the low concentrations used, the target enrichment primers will generate target specific sequences with super primer binding tags incorporated therein. These super primer binding tags will generate super primer binding sequences for use by the target amplification primers for exponential amplification of the target sequences. The resulting amplified target sequences can be detected using target specific reporter (also referred to as detection) oligonucleotide as described below.

The result of the Tem-PCR amplification procedure is the specific enrichment of target sequences by the target enrichment primers, and the subsequent exponential amplification of the target sequences by the target amplification primers utilizing the super primer binding sites provided by the copying of amplification products containing the super primer binding tags. Since the exponential amplification of the each of the target sequences is carried out by one or more sets of target amplification primers the amplification conditions can be standardized and optimized taking into account only the target amplification primers. Therefore, incompatibility of the exponential amplification reaction conditions is not of concern as is the case for multiplex amplification methods currently known in the art.

The Tem-PCR amplification strategy produces decreased background since only the target amplification primers are present in high concentrations. As a result, the occurrence of primer dimmer formation and related phenomenon are reduced. Further adding to the reduced background, since only the super primers (or 1 of each set of super primers) are conjugated with a means for detection, even if primer dimmer formation occurs, it will not be detected by the reporter oligonucleotides. The reduced background will decrease the chance of false positive diagnosis.

The ratios of the target enrichment primers (F_{out}, F_{in}, R_{out}, and R_{in}) used in the Tem-PCR amplification method may be varied. Different pathogen genomes may have different target enrichment primer requirements. As discussed previously, some disease agents and secondary disease agents may have DNA genomes or RNA genomes (positive or negative strand). In

addition, the concentration of target amplification primers may also be varied, especially if only 1 of the super primers is conjugated to a means for detection. Multiplex amplification where at least one of the target enrichment primers or super primers is used at a ratio different than 1:1 is referred to as asymmetric multiplex amplification.

In an experiment conducted using various target enrichment primer and target amplification primer ratios it was determined that increased amplification of the target sequences derived from negative strand RNA genomes occurred at a range of primer concentrations. In one embodiment, the concentration of F_{out} was 1.0 to 8-fold greater than the concentration of the remaining target enrichment primers. An exemplary ratio would be 4:1:1:1 for F_{out}:F_{in}:R_{out}:R_{in}. For negative stranded RNA viruses, the amount of F_{out} may be increased over the ratio of the remaining nested primers in order to provide for initial amplification of a positive RNA strand complementary to the genomic negative strand RNA. In one embodiment, increased amplification was observed when the concentrations of target amplification primers are about 1.25 to 32-fold greater than the concentration of F_{out}. An exemplary ratio would be 4:1:1:1:10:40 for F_{out}:F_{in}:R_{out}:R_{in}:FSP:RSP, respectively. The ratios of the individual target amplificaiton primers can also be varied with respect one another. This asymmetric variation has been shown to provide increased sensitivity in the detection step (see Table 4). In one embodiment, the super primer incorporating the means for detection is added in a higher concentration that the other super primer. In one embodiment the RSP contains the means for detection and is added at a higher concentration. Increased amplification of the target sequences was observed when the concentrations of RSP were 1.25 to 16-fold greater than the concentration of FSP. An exemplary ratio would be 10:40 for FSP:RSP, respectively.

For amplification of target sequences from disease agents and secondary disease agents with positive stranded RNA genomes, additional primer ratios may be employed. In one embodiment, the concentration of R_{out} was 1.0 to 8-fold greater than the concentration of the remaining nested primers. An exemplary ratio would be 1:1:4:1 for F_{out}:F_{in}:R_{out}:R_{in}. For positive stranded RNA viruses, the amount of R_{out} may be increased over the ratio of the remaining target enrichment primers in order to provide for increased amplification the RNA strand containing the target sequence. In one embodiment, increased amplification was observed when the concentrations of target amplification primers are about 1.25 to 32-fold greater than the concentration of R_{out}. An exemplary ratio would be 1:1:4:1:10:40 for F_{out}:F_{in}:R_{out}:R_{in}:FSP:RSP, respectively. The ratios of the individual target amplification primers can also be varied with respect one another. This asymmetric variation has been shown to provide increased sensitivity in the detection step (see Table 4). In one embodiment, the super primer incorporating the means for detection is added in a higher concentration that the other super primer. In one embodiment

the RSP contains the means for detection and is added at a higher concentration. Increased amplification of the target sequences was observed when the concentrations of RSP were 1.25 to 16-fold greater than the concentration of FSP. An exemplary ratio would be 10:40 for FSP:RSP, respectively.

For amplification of target sequences from disease agents and secondary disease agents with DNA genomes, additional primer ratios may be employed. In one embodiment, the concentration of the target enrichment primers are essentially equivalent. In an alternate embodiment, the concentration of one of F_{out} or R_{out} was 1.0 to 4-fold greater than the concentration of the remaining nested primers. An exemplary ratio would be 1:1:1:1 for F_{out}:F_{in}:R_{out}:R_{in}. In one embodiment, increased amplification was observed when the concentrations of target amplification primers are about 1.25 to 128-fold greater than the concentration of R_{out}. An exemplary ratio would be 1:1:1:1:10:40 for F_{out}:F_{in}:R_{out}:R_{in}:FSP:RSP, respectively. The ratios of the individual target amplification primers can also be varied with respect one another. This asymmetric variation has been shown to provide increased sensitivity in the detection step (see Table 4). In one embodiment, the super primer incorporating the means for detection is added in a higher concentration that the other super primer. In one embodiment the RSP contains the means for detection and is added at a higher concentration. Increased amplification of the target sequences was observed when the concentrations of RSP were 1.25 to 16-fold greater than the concentration of FSP. An exemplary ratio would be 10:40 for FSP:RSP, respectively.

In the embodiment where only 1 pair of target enrichment primers is utilized, the primers may be used at the different ratios discussed above (including the discussed target amplification primer ratios), with the ratios of the F_{out} or R_{out} primers above serving as the ratios for the corresponding primers in the 1 pair of target enrichment primers The concentration of the target enrichment primers are essentially equivalent.

Without being bound to alternate explanations, it is possible that as the concentration of target enrichment primers increase, target sequences are amplified without a means for detection and are able to complete for binding to the reporter oligonucleotide with the target sequences comprising a means for detection that are amplified by the FSP and/or RSP. The asymmetric amplification utilizing the target amplification primers may also increase amplification efficiency and sensitivity when the super primer containing the means for detection is used at a higher concentration because more amplification products containing the target sequence and the means for detection are produced and available for detection. Table 1 provides one set of exemplary ratios for the concentration of F_{out}, F_{in}, R_{out}, and R_{in} as well as FSP and RSP.

As discussed above, the target amplification primers are used for the exponential amplification of each target sequence. The sequences of the target amplification primers are selected so they do not share obvious homology with any known GenBank sequences. In addition, the sequence of the target amplification primers is selected so they share a comparable Tm on binding to the super primer binding sites in the amplification products to provide efficient amplification reactions. Finally, the sequence of the target amplification primers may be selected such that their priming capabilities for thermal stable DNA polymerases maybe superior to the target enrichment primers which are specific for each target sequence to be amplified.

The multiplex amplification is performed as per standard procedures known in the art. In one embodiment, RT-PCR or PCR is used as the amplification step. The PCR enzyme may be an enzyme with both a reverse transcription and polymerase function, such as Taq polymerase. Furthermore, the PCR enzyme may be capable of "hot start" reactions as is known in the art. The conditions for RT-PCR or PCR are known in the art. However, the applicants have produced an optimized set of RT-PCR and PCR conditions that are designed for use with the TemPCR method. The exact times and temperatures may be varied to accomplish the objects as discussed below as would be known to one of skill in the art. In one embodiment, the RT-PCR or PCR conditions are as follows. The method below comprises a set of first amplification conditions for a first amplification reaction that serves a target enrichment function and a second amplification reaction that comprises a target amplification function. The samples containing the reagents for amplification were placed in a thermocycler programmed as follows: (i) reverse transcription- 30 minutes at 50^0C; (ii) initial PCR activation- 15 minutes at 95^0C; (iii) first amplification reaction comprising a first 3-step cycling (target enrichment)- 0.5 to 1 minute at 92-94^0C, 1 to 2.5 minutes at 50-55^0C and 0.5 to 1 minute at 70-72^0C; for at least 2 complete cycles, preferably for 10-15 complete cycles; (iv) second amplification reaction comprising a second 3-step cycling (target amplification)- 15 to 30 seconds at 92-94^0C, 15 to 30 seconds at 50-55^0C and 15 to 30 second at 70-72^0C; for at least 2 complete cycles, preferably for 10-40 complete cycles; and (v) final extension- 3 minutes at 72^0C.

Step (i) allows for the reverse transcription reaction to occur in the cases where the nucleic acid of the disease agent and/or secondary disease agent is not DNA. Step (ii) allows for activation of the PCR enzyme function. This technique is known as "hot start" PCR, where at lower temperatures (50^0C) the PCR enzyme function is only competent to carry out the reverse transcription reaction, but at higher temperatures the PCR enzyme function is capable of polymerase function. Incubation of the PCR enzyme function is required to relieve the inhibition of the polymerase function. The first amplification in step (iii) is designed to allow the target enrichment primers to hybridize to their targets and prime the target enrichment step. As can be

seen, additional time is allowed for the target enrichment primers to hybridize to their targets (2.5 minutes at 50-55^{0}C as compared to 15 seconds 50-55^{0}C in step (iv)). Increased times are used since the target enrichment primers are used at lower concentrations. The target amplification reaction in step (iv) is the exponential amplification phase using the target amplification primers (which are at high concentration). Since the target amplification primers are present at high concentrations, increased hybridization times between the target amplification primers and targets are not required. Step (v) is the final extension step to generate full double stranded amplification products. Step (v) is optional and need not be included.

In an alternate embodiment, the RT-PCR or PCR conditions are as follows. The reaction tubes containing the reagents for amplification were placed in a thermocycler programmed as follows: (i) reverse transcription- 30 minutes at 50^{0}C; (ii) initial PCR activation- 15 minutes at 95^{0}C; (iii) first amplification reaction comprising a first 3-step cycling (target enrichment)- 0.5 to 1 minute at 92-94^{0}C, 1-2.5 minutes at 50-55^{0}C and 0.5 to 1 minute at 70-72^{0}C; for at least 2 complete cycles, preferably for 10-15 complete cycles; and a second 2-step cycling (selective amplification)- 15 to 30 seconds at 92-94^{0}C, 1 to 2 minutes at 70-72^{0}C; for at least 2 complete cycles, preferably 4-8 complete cycles; (iv) second amplification reaction comprising a third 3-step cycling (target amplification)- 15 to 30 seconds at 94^{0}C, 15 to 30 seconds at 50-55^{0}C and 15 to 30 second at 72^{0}C; for at least 2 complete cycles, preferably for 10-40 complete cycles; and (v) final extension- 3 minutes at 72^{0}C.

In this embodiment, the functions of the various steps are as described above. However, the first amplification reaction comprises an additional 3-step cycling procedure has been added to increase the production of amplification products incorporating the super primer binding tags, which ultimately give rise to amplification products containing the super primer binding sites. In the added 3-step cycling prodecure, the hybridization temperature in increased to 70-72^{0}C (as compared to 50-55^{0}C in step (iii)). The hybridization time is also increased as discussed above to give the low concentration target enrichment primers an opportunity to hybridize to their targets. This increased hybridization temperature endures that only target enrichment primers of a certain length are stable enough to hybridize to their targets. In this embodiment, the F_{out} and R_{out} primers are selected to have a length of 20 nucleotides or less, while the F_{in} and R_{in} primers (which in this embodiment comprise the super primer binding tags) are at least 30 to 40 nucleotides in length. Therefore, the F_{out} and R_{out} primers are not thermally stable at 70-72^{0}C, meaning they will not hybridize to their targets. However, the increased length of the F_{in} and R_{in} primers ensures that these primers are thermally stable at 70-72^{0}C and will hybridize to their targets. In the selective amplification steps, the F_{in} and R_{in} primers hybridize along their entire length (40 nucleotides) to complementary sequences in the initial set of amplification products

created by the amplification in the opposite direction of initial amplification products incorporating the F_{in} and R_{in} primers. (in the target enrichment step, the F_{in} and R_{in} primers The F_{in} and R_{in} primers produce an increased amount of amplification products containing the super primer binding tags,. which increases the amount of exponential amplification in step (v). Therefore, step (iv) is a selective amplification step biased towards producing amplification products containing the super primer binding tags. This selective amplification is accomplished without the need to engineer higher GC content or without the need to use modified nucleotides to increase thermal stability. The amplification products of step (iv) are then subject to exponential amplification as described above.

Multiplex Detection

A robust target sequence multiplex amplification method provides the basis for a sensitive and specific multiplex detection. The multiplex detection method used in MAS complements the multiplex amplification to provide balanced sensitivity and specificity in detection of amplified target sequences. Sensitivity and specificity are important issues for a successful diagnostic test or differential diagnostic test.

The multiplex detection method for use in MAS can be either a direct detection or an indirect multiplex detection method. The nature of the target sequences will in part determine whether direct or indirect detection is used. For example, if the target sequences do not share extensive homology with one another, the required sensitivity and specificity may be obtained by directly hybridizing the reporter oligonucleotide to the target sequences. For example, in diagnostic test for a disease agents or differential diagnostic test for disease agents and at least one secondary disease agent, the multiplex amplification may be designed so that each of the target sequences amplified from the disease agent and the secondary disease agent do not share extensive homology so that direct detection may be used. However, if the target sequences amplified do share extensive homology with one another, additional steps may be incorporated in the multiplex detection step to add additional discrimination between the homologous sequences so that the required sensitivity and specificity are obtained. For example, in a diagnostic test to determine the presence of one or more single nucleotide polymorphisms in the alleles of an individual, direct hybridization may not be able to provide the required allelic discrimination required to specifically detect target sequences differing by only one nucleotide. In this case, an indirect detection method may be used to add additional discriminatory power.

FIG. 2 shows an embodiment of the direct detection method. Amplified nucleic acid (1) containing a target sequence (6) from a multiplex amplification reaction is provided for detection (the amplification process may be the Tem-PCR process described herein). Reporter

oligonucleotides (2) comprising a hybridization domain (3) specific for a known target sequence (6) are provided and are conjugated to a means for first signal generation (4). The means for first signal generation is capable of producing a detectable first signal. The means for first signal generation (4) may be varied depending on the technology platform employed in the detection step. For example, if the Luminex platform is employed as the technology platform in the detection step, the means for first signal generation (4) may be an internally color coded, spectrally addressable microsphere as shown in FIG. 2. Variations to the direct detection methods described below are described in co-pending U.S. application serial number 10/284,656, with the implementation of such variations being obvious to one of ordinary skill in the art.

The reporter oligonucleotides (2) are designed to hybridize specifically to a specific target sequence (6) provided by the multiplex amplification step discussed above. The specificity of the hybridization between the reporter oligonucleotide (2) and the target sequence (6) can be adjusted by increasing or decreasing the length of the hybridization domain (3) of the reporter oligonucleotide. In general, a shorter hybridization domain (3) will give increased specificity and differentiation as a mismatch between the target sequence (6) and the hybridization domain (3) will have a significant impact on the hybridization efficiency. A longer hybridization domain (3) will provide less specificity but greater hybridization efficiency and therefore increased sensitivity. The nature of the target sequence (6) will influence the composition of the hybridization domain (3). One of ordinary skill in the art would be able to alter the parameters of the hybridization domain (3) to achieve the desired specificity and sensitivity of binding of the hybridization domain (3) to the target sequence (6). In one embodiment, the hybridization domain (3) is 10 to 50 bp in length. In an alternate embodiment, the hybridization domain (3) is 20-40 bp in length. In yet another alternate embodiment the hybridization domain (3) is 15 to 25 bp in length.

Each reporter oligonucleotide (2), which specifically hybridizes to a known target sequence (6) via the hybridization domain (3), will be associated with a known means for first signal generation (4) (such as a color coded bead). Therefore, by determining the identity of the means for first signal generation (4), the identity of the target sequence (6) can be determined, and therefore the identity of the disease agent or secondary disease agent can be determined.

Amplified nucleic acid (1) containing a target sequence (6) may be denatured before or during the multiplex detection step, if desired. Denaturation is not required. The TemPCR amplification reaction using asymmetric amplification conditions (such as RSP being present in greater concentrations that FSP, such as a 40:10 ratio) produces sufficient single stranded amplification products for the detection reaction that denaturation is not a required step. Denaturation, in this and other steps referred to in this disclosure, may occur by heating to a

sufficient temperature or by chemical means (such as, but not limited to, the addition of agents such as 5N NaOH). For the following embodiment, denaturation of the amplified nucleic acid (1) containing a target sequence (6) was not used. However, the principles embodied in the following steps will not differ if the denaturation step is added.

The reporter oligonucleotides (2) are added to the denatured amplified nucleic acid (1) in appropriate hybridization buffer (such as 1X TMAC or 1X TE). The addition of the reporter oligonucleotides (2) may occur before or after denaturation (if employed). The reporter oligonucleotides (2) bind the target sequences (6) on the amplified nucleic acid (1) through the hybridization domain (3) forming a nucleic acid-reporter oligonucleotide complex (I). Hybridization conditions as are known in the art, such as by incubation at 52^{0}C for 15 minutes, may be used. After hybridization is complete, complex I is isolated, such as by centrifugation, and the unbound reporter oligonucleotides (2') and unbound amplified nucleic acid (1') are removed. Complex 1 may then be subject to detection using an appropriate detection platform. In one embodiment, the means for first signal generation (4) is an internally color coded spectrally addressable bead (Luminex) and the Luminex platform is used for detection. The Luminex platform stimulates the means for first signal generation (4) to produce a detectable first signal. The first signal is recorded and interpreted. The means for first signal generation (4) and the produced first signal are used to determine the identity of the target sequence (6) bound by the reporter oligonucleotide (2). Once the target sequence (6) has been identified, the identity of the disease agent or secondary disease agent detected is known.

However, the amplified nucleic acid (1) may contain a means for detection (8) provided by at least one of the FSP or RSP. This means for detection (8) may be as described above and may be directly or indirectly conjugated to the amplified nucleic acid (1). In FIG. 2, a biotin molecule provided by one of the target amplification primers is illustrated as the means for detection (8). The means for detection (8) may be used to incorporate a means for second signal generation (10) which is capable of generating a detectable second signal. In one embodiment, the means for second signal generation (10) may be a streptavidin molecule bound to a detectable label, such as a fluorescent label, a chemical label, an enzymatic label or a radiolabel. Suitable fluorescent labels, include but are not limited to, PE or Cy-3. In one embodiment, the means for second signal generation (10) is a streptavidin-PE complex. In an alternate embodiment, the means for second signal generation (10) may be directly conjugated to the amplified nucleic acid product as a component of the detecting means. The means for second signal generation (10) is added to the nucleic acid-reporter oligonucleotide complex and incubated to allow for binding to form complex (II). In one embodiment, the incubation is for 5 minutes at 52^{0}C, although other times and temperatures may be used as is known in the art.

The complex (II) may then be analyzed using an appropriate platform. In one embodiment, the means for first signal generation (4) is an internally color coded spectrally addressable bead (Luminex) and the means for second signal generation (10) is a fluorescent PE label. In this embodiment, the Luminex platform is used for detection. The Luminex platform stimulates the means for first (4) second (10) signal generation to produce a detectable first and second signal, respectively. The first and second signal generating means are selected such that the first and second signals can each be detected in presence of the other. The first and second signal are recorded and interpreted. The means for first signal generation and the produced first signal are used to determine the identity of the target sequence (6) bound by the reporter oligonucleotide (2). The means for second signal generation (10) and the second signal are used to confirm the presence of the target sequence (6) in combination with the reporter oligonucleotide (2) (to prevent signal generation from any free reporter oligonucleotide (2)). Once the target sequence (6a) has been identified, the identity of the disease agent or secondary disease agent detected is known. However, it should be noted that complex I may be subject to detection as described above

Additional technology platforms may be used in the direct detection step as well. In an alternate embodiment, the first signal generating means on the reporter oligonucleotide may produce a detectable physical first signal when placed in an appropriate apparatus for detection and analysis. The physical signal may be a color change, an emission of a given wavelength of light upon excitation or a change in the electrical properties, such as conductivity, or a change in the electromagnetic or chemical properties. Other physical signals may also be used. In one embodiment, the means for first signal generation may be spatial in nature, such as location on a solid support. For example, reporter oligonucleotide may be spatially resolved at a known location on the collecting means such as a chip or other solid support. Therefore, the first signal is spatial in nature. When the amplified products containing the target sequence hybridize to a reporter oligonucleotide, the binding may be determined by the presence of the second signal generated by the second signal generating means on the amplified nucleic acid. By detecting the spatial location (first signal) of the second signal, the identity of the target sequence and the disease agent/secondary disease agent is determined. In an alternate embodiment, the reporter oligonucleotide may be coupled to an additional first signal generating means so that two first signals are produced, one spatial and one non-spatial. Therefore, the second signal produced by the second signal generating means will signal binding of the target nucleic acid to the reporter oligonucleotide. In an alternate embodiment, the biding of the target sequence to the reporter oligonucleotide may produce a detectable change in the characteristics of the reporter oligonucleotide (including but not limited to changes in electrical properties such as

conductivity). This change in characteristics would then serve as the second signal.

In one embodiment of the indirect detection protocol, a novel method termed ROCASH (Reporter Oligo Capturing After Specific Hybridization) is used. The ROCASH method is described in co-pending U.S. application serial number 10/284,656, which is hereby incorporated by reference as if fully set forth herein. For sake of clarity, one embodiment of the ROCASH method using the Luminex X-Map technology is described. Other methods of indirect detection may also be used as is known in the art. It is understood that variations of the ROCASH method may be incorporated as described in the co-pending U.S. application serial number 10/284,656. Unlike the prior art methods, in the ROCASH method the specificity of hybridization between a reporter oligonucleotide and the target sequence and the sensitivity of the detection of the target sequence are provided by different nucleic acid sequences of the reporter oligonucleotide hybridizing in different steps of the ROCASH method. Therefore, the conditions for these critical steps may be optimized independently of each other to provide for increased specificity and sensitivity in the detection step.

The Luminex xMAP technology and related technologies are described in the art and in US Patent Nos. 6,524,473, 6,514,295, 6,449,562, 6,411,904, 6,366,354, 6,268,222, 6,139,800, 6,057,107, 6,046,807 and 5,736,330. The xMAP technology uses a plurality of internally color-coded microspheres covalently bound to target specific capturing reagents (termed cRTs, as defined below). Such capturing reagents may be oligonucleotides (as in the case of cRTs described below), but may also be polypeptides or chemical moieties designed to interact specifically with the region tags. When alternate capturing reagents are used, the region tags on the reporter oligonucleotides may altered to provide a complementary binding partner. The internal color coding generates a unique signal for each set of beads on excitation by the Luminex platform.

The ROCASH method may be considered to comprise two primary components: (i) a reporter oligonucleotide; and (ii) a means for collection. The reporter oligonucleotides are designed to bind specifically to the target sequence provided by the multiplex amplification step discussed above. The reporter oligonucleotides comprise a hybridization domain of variable length designed to hybridize to the target sequence (see discussion above regarding the effect of the length of the hybridization domain on specificity and sensitivity), a nucleic acid sequence termed the region tag for hybridization to the means for collection, and a means for first signal generation. In one embodiment, the region tags on the reporter oligonucleotides are unique for each hybridization domain. In other words, a reporter oligonucleotide having a hybridization domain that binds to target sequence "A" and a reporter oligonucleotide having a hybridization domain that binds to target sequence "B" will have different region tags. The means for

collection comprises and a plurality of capturing reagents, in this embodiment termed cRTs (complementary region tags) and a means for second signal generation. cRTs are nucleic acid sequences that are complementary to the region tags contained in the reporter oligonucleotides. The means for first and second signal generation may vary depending on the nature of the technology employed in the detection platform. When using the Luminex X-Map technology, the means for first signal generation may be a fluorescent tag, such as PE or Cy-3, and the means for second signal generation may be an internally color coded, spectrally addressable microsphere. Through interaction of the region tags of the reporter oligonucleotide and the cRTs of the means for collection, each reporter oligonucleotide (which is specific for a known target sequence by virtue of the hybridization domain) will be associated with a known means for second signal generation (such as a color coded Luminex bead). Therefore, by determining the identity of the means for second signal generation, the identity of the target sequence can be determined, which will allow the identification of the disease agent or secondary disease agent. Therefore, the specificity is achieved by the hybridization between the hybridization domain of the reporter oligonucleotide and the target sequence (note that specificity may be altered by decreasing or increasing the length of the hybridization domain) and the sensitivity is determined by the hybridization between the region tag of the reporter oligonucleotide and the cRT of the means for collection. A means for purification may also be used that is designed to interact with the amplified nucleic acid sequences and aids in the removal of the unused amplified target nucleic acids as discussed below.

One embodiment of the ROCASH procedure is illustrated in FIG. 3. Amplified nucleic acid (1a) containing a target sequence (6a) from a multiplex amplification reaction is provided for detection (the amplification process may be the Tem-PCR process described herein). The amplified nucleic acid (1a) containing the target sequence (6a) may be denatured if desired. The denaturation step is optional however. FIG. 3 shows the amplified nucleic acid (1a) after a denaturation step. As discussed above, the reverse strand of the amplified nucleic acid will contain a means for detection (8a) provided by at least one of the FSP or RSP. This means for detection (8a) may be a biotin molecule attached to the FSP and/or the RSP or other such means as is known in the art as discussed above. The means for purification (9a) is selected to interact with the means for detection (8a). In one embodiment, the means for purification (9a) is a magnetic bead (such as the Dynal270 magnetic bead) conjugated to streptavidin and the means for detection (8a) is a biotin label. The means for purification (9a) is added to the amplified nucleic acid (1a) where it binds the means for detection (8a) to form a nucleic acid-means for purification complex (complex I). Complex I is washed to remove the unbound nucleic acid and the remaining components and isolated using separation techniques known in the art and

compatible with the means for purification. In the case where the means for purification is a magnetic bead, magnetic separation techniques may be used. Hybridization buffer, such as 1X TMAC or 1X TE, is added to the complex I to prepare for hybridization.

The reporter oligonucleotides (2a) are then added to the complex I. The reporter oligonucleotides may be added at prior steps, however, if desired, either before or after the denaturation of the amplified nucleic acid. The reporter oligonucleotides (2a) bind the target sequences through the hybridization domain (3a) forming a means for purification-nucleic acid-reporter oligonucleotide complex (complex II). The reporter oligonucleotides (2a) also comprise a region tag (12a) and a means for first signal generation (4a). Hybridization may occur as is known in the art, such as by incubation at 52^0C for 15 minutes. After binding, complex II may be washed (such as in warm 1X SSC) and isolated using appropriate separation techniques as discussed above. After removal of unbound reporter oligonucleotides, complex (II) is subject to denaturation conditions to release the reporter oligonucleotide (2a) from target sequences (6a). The nucleic acid-means for purification complex is removed by appropriate separation techniques as discussed above.

The means for collection (20a) is mixed with the reporter oligonucleotides (2a) remaining in solution. The means for collection comprises a cRT (22a) and a means for second signal generation (10a). The cRT (22a) of the means for collection (20a) interacts with the region tags (12a) of the reporter oligonucleotides forming a collecting means-reporter oligonucleotide complex (complex III). The cRTs (22a) and region tags (12a) are selected so that each cRT/region tag complementary pair has a similar T_m for binding to one another. Therefore, the hybridization conditions for each region tag (12a) to it complementary cRT (22a) are universal. This allows for increased sensitivity of detection, particularly in a multiplex detection setting, since one set of hybridization conditions are used. Hybridization conditions as described above may be used as well as other hybridization conditions as are known in the art. Complex (III) is then isolated and analyzed using an appropriate detection platform

In one embodiment, the means for first signal generation (4a) is a fluorescent PE label and the means for second signal generation (10a) is an internally color coded spectrally addressable bead (Luminex). In this embodiment, the Luminex platform is used for detection. The Luminex platform stimulates the means for first (4a) second (10a) signal generation to produce a detectable first and second signal, respectively. The first and second signal are recorded and interpreted. The first and second signal generating means are selected such that the first and second signals can each be detected in presence of the other. The means for second signal generation and the produced second signal are used to determine the identity of the target sequence (6a) bound by the reporter oligonucleotide (2a). The means for first signal generation (10a) and the second

signal are used to indirectly confirm the presence of the target sequence (6a) (to prevent signal generation from any free means for collection 10a). Once the target sequence (6a) has been identified, the identity of the disease agent or secondary disease agent detected.

Differential Diagnosis of Severe Acute Respiratory Syndrome (SARS) Using an Embodiment of MAS

Below are several examples of the MAS utilizing the teaching of the present disclosure to provide a differential diagnostic assay for SARS (in these examples SARS-CoV). In this example, SARS is the disease agent and the secondary disease agent includes one or more of the following: respiratory syncytial virus (RSV) A and B, parainfluenza virus (PIV) 1 and 3, influenza (INF) A and B, adenovirus strains (ADV), *C. pneumoniae* and *M. pneumoniae*, enterovirus (ENT), and enterovirus types such as coxsackie virus A (CVA), coxsackie virus B (CVB), rhinovirus (RhV), and echovirus (EV). As discussed above, these examples are provided as exemplary in nature and are not meant to limit the present disclosure to the disclosed differential diagnosis as other target sequences from other disease agents and secondary disease agents may be amplified and detected.

Tables 2 and 3 lists the sequence of the target enrichment primers (designated F_O, F_I, R_O, and R_I for F_{out}, F_{in}, R_{out} and R_{in}, respectively, as such terms are defined herein) used in the TemPCR method to amplify target sequences from the disease agent and secondary disease agents, as well as the nucleic acid sequence of the reporter oligonucleotide (designated De). In Tables 2 and 3 the F_i and R_i primers contained the super primer binding tag. Table 2 also provides the GenBank accession numbers for the nucleic acid sequence of the disease agent and secondary disease agents as well as the region of nucleic acid bound by the primers and the reporter oligonucleotide. The sequence of the target amplification primers (FSP and RSP) are provided at the bottom of Tables 2 and 3.

In these examples, three target sequences from the SARS viral genome were amplified and detected. The SARS virus is known to have a high mutation rate. If mutations occur at the primer binding sites or at the detection hybridization site, a false negative result may be introduced. Detecting three targets from the SARS pathogen increases detection sensitivity and reduces false negative results. For the secondary disease agents, one region of nucleic acid from each of the respective nucleic acids was amplified and detected. The target enrichment primers listed in Table 2, in some cases, may be used to detect multiple strains of a pathogen. For example, the target enrichment primers used to amplify influenza A produce amplified nucleic acid containing a target sequence sufficient for the detection and differentiation (using the appropriate detection oligonucleotides) of a variety of strains, including, but not limited to,

H1N1, H1N2, H2N2, H3N2, H4N6, H5N1 and H9N2. The target enrichment primers used to amplify influenza B produce amplified nucleic acid containing a target sequence sufficient for the detection and differentiation (using the appropriate detection oligonucleotides) of a variety of strains, including but not limited to, Lee40, Memphis 97, Saya 99 and Taiwan 99. The primers used to amplify the adenovirus produce amplified nucleic acid containing a target sequence sufficient for the detection and differentiation (using the appropriate detection oligonucleotides) of adenovirus strains 3, 4, 7, 14, and 21. In each of the above cases, detection oligonucleotides specific for each strain can be used in the detection step to identify a particular strain. The RSP is labeled with a means for detection, such as biotin (other labeling molecules may be used as discussed above).

The target enrichment primers listed in Table 3 also, in some cases, may be used to detect multiple strains of a pathogen. Table 4 shows the genes amplified from the various pathogens by the target enrichment primers shown in Table 3. A nonstructural gene (NS) was selected as the amplification from the Influenza A viruses. Conserved regions were selected for the design of the 4 target enrichment primers and produce amplified nucleic acid containing a target sequence sufficient for the detection and differentiation (using the appropriate detection oligonucleotides) of a variety of strains including, but not limited to, H1N1, H1N2, H2N2, H3N2, H3N8, H4N6, H4N8, H5N1, H5N2, H5N3, H6N1, H6N2, H6N4, H7N1, H7N2, H7N3, H7N7, H7N8, H9N2, H10N5, H11N1, H11N8, and H11N9 (which includes the currently circulating avian influenza A strain, H5N1). A nonstructural gene was also selected as the target for amplification and detection of Influenza B virus. There are at least 51 known serotypes of adenoviruses. Conserved regions in the hexon gene were selected for the design of the series of target enrichment primers and produce amplified nucleic acid containing a target sequence sufficient for the detection and differentiation (using the appropriate detection oligonucleotides) of a variety of strains including, but not limited to, Serotypes 3, 4, 7, 14 and 21 (which includes those serotypes commonly associated with respiratory infections). The detection oligonucleotide ADV3-3De detects strains 3, 7 and 21, ADV4-3De detects strain 4 and Adv14-3De detects strain 14. Conserved sequences from the 5' UTR region of various enteroviruses species and family members were selected for amplification. Enteroviruses and rhinoviruses are members of the Picornaviridae family. Enteroviruses also include different genera such as coxsackie viruses and echoviruses. These viruses are all associated with respiratory infections. For the detection of various members of the enterovirus class, a series of target enrichment primers were designed that produces amplified nucleic acid containing a target sequence sufficient for the detection and differentiation (using the appropriate detection oligonucleotides) of a variety of strains of: rhinovirus including, but not limited to, Ts, 1a, 1b, 2, 9, 14, 15, 16, 39, 49, 50, 85, and 89; coxackie virus A, including, but not

limited to, A21 and A24; coxackie virus B including, but not limited to, B4 and B5; and echovirus including, but not limited to, 11, 20, and 25. The detection oligonucleotide Rhv2 is specific for the detection of the rhinovirus, while the detection oligonucleotide CVE2 detects coxackie virus A and B and echovirus.

Conserved sequences from the nucleocapsid protein gene (N gene) were selected amplification from the Parainfluenza type 1 and 3 viruses. Nonstructural gene sequences were selected as targets for Respiratory syncytial viruses A and B. To reduce background amplification and false positive detections, rRNA genes were not selected for amplification from the bacteria species listed. Instead, the cytadhesin P1 gene from Mycoplasma pneumoniae, and the Uridine Kinase gene from Chlamydia pneumoniae were selected for amplification. The RSP is labeled with a means for detection, such as biotin (other labeling molecules may be used as discussed above).

The primer sequences used in the present detection method may be varied as discussed in more detail above. In addition, other agents may be included for detection in the method disclosed by designing primers specific for the agents. Such modification may be desirable if new agents are discovered that cause a SARS-like disease state or if additional SARS variants are isolated.

Example 1

In this example, nucleic acid sequences from each disease agent and the secondary disease agents were obtained and isolated as described herein. The nucleic acid from each agent was amplified by the Tem-PCR method described herein. The nucleic acid for each agent was placed in a separate reaction tube and the complete mix of primers specified in Table 2 was added along with reagents for RT-PCR. A separate reaction was carried out for each of the three primer ratios specified in Table 5 for each agent. While any procedure known in the art for RT-PCR may be used, the following procedure was used in the example. A master RT-PCR mix was prepared containing 5X RT-PCR buffer, deoxy nucleoside triphosphate (dNTP) mix (containing 400 uM of each dNTP), the complete mix of primers shown in Table 2 at the ratios specified in Table 5, and RT-PCR enzyme preparation. An RNAse inhibitor may also be added at a concentration of 5-10 units/reaction if desired. The nucleic acid sequences or portions thereof containing the target sequences from each disease agent and secondary disease agent sufficient for amplification were added to individual reaction tubes containing the RT-PCR master mix. A RT-PCR blank was prepared by adding RNAse free water to one reaction tube in place of nucleic acid template. The reaction tubes were placed in a thermocycler programmed as follows: (i) reverse transcription- 30 minutes at 50^0C; (ii) initial PCR activation- 15 minutes at 95^0C; (iii) first amplification reaction comprising a first 3-step cycling (target enrichment)- 1 minute at 94^0C, 2.5 minutes at 55^0C and 1

minute at 72°C for 10 complete cycles; (iv) second 3-step cycling (target amplification)- 30 seconds at 94°C, 15 seconds at 55°C and 15 second at 72°C for 40 complete cycles; and (v) final extension- 3 minutes at 72°C.

An aliquot of the amplified products were subject to detection using the direct detection method as described above and illustrated in FIG. 2. In this example, detection oligonucleotides (as listed in Table 2) specific for a target sequence amplified in each agent to be detected were coupled to Luminex microspheres. An aliquot of the amplified products were added, in separate tubes, to a reaction mixture containing 1X TE, hybridization buffer and an amount of each detection oligonucleotide sufficient to generate a detectable signal. In this example, detection oligonucleotide for each agent in Table 2 was present in each reaction containing the amplified nucleic acid products. For the detection oligonucleotide negative control, 1X TE was added in place of the amplified products. The samples were immediately placed at 52°C for 15 minutes for hybridization between the detection oligonucleotide and the target sequence. The samples were centrifuged to remove unbound detection oligonucleotide and nucleic acid to which the detection oligonucleotides had not bound. The samples were then subject to detection using the Luminex platform. The Luminex platform stimulated the microsphere conjugated to the detection oligonucleotide (the first signal generating means) to produce a detectable signal. In this example, a second means for detection was not included, however, a second signal may be incorporated as discussed above.

The results are presented in Table 3. The rows represent the identity of the disease agent or secondary disease agent whose nucleic acid was used in the initial amplification reaction and the ratio of the primers used in the amplification step. The columns represent the detection oligonucleotides used in the detection step. For example, row 5 (designated RSVB 4:1:1:1:8:16) indicates the nucleic acid of respiratory syncytial virus type B was used in the amplification reaction and the nested primers and super primers were used in a ratio of 4:1:1:1:8:16. Row 2 (designated RSVB) indicated the detection oligonucleotide specific for the respiratory syncytial virus type B target sequence was used in the detection step. Column 1 (designated Lum Blank) represents a detection oligonucleotide negative control where the amplified products omitted. Rows 3-5 (designated Blank 4:1:8:16, Blank 4:1:8:24 and Blank 4:1:8:32, respectively) represent amplification negative controls where the specific nucleic acid sequence was omitted but the primers for amplification of each specific target sequence were included at the indicated ratios.

As can be seen in Table 4, the detection oligonucleotides specific for a given disease agent or secondary disease agent specifically bound to the nucleic acid from that disease agent or secondary disease agent in the presence of multiple amplified target sequences. This result indicates that the target enrichment primers amplified the correct target nucleic acid sequence in

the presence of multiple primer sets not specific for the target sequence and that the target amplification primers (FSP and RSP) function correctly to amplify the target sequence as described and the appropriate detection oligonucleotides are capable of hybridizing to the target sequence being detected. The detection oligonucleotide negative control and amplification negative controls provided background readings and showed no excessive background signal in all samples tested.

As one example, the detection oligonucleotide specific for RSVB bound specifically to the target sequences derived only from RSVB and no other agents. In addition, the level of detection increased as the concentration of RSP was increased, indicating that asymmetric amplification can increase sensitivity of the detection step. Furthermore, in the case of adenovirus, Table 4 indicates that the target enrichment primers and target amplification primers amplified the correct adenovirus target sequence and that the specific detection oligonucleotides were capable of discriminating between the adenoviral strains.

Example 2

In this example, nucleic acid sequences from each disease agent and the secondary disease agents were obtained and isolated as described herein. The nucleic acid from each agent was amplified by the Tem-PCR method described herein. The nucleic acid for each agent was placed in a separate reaction tube and the complete mix of primers specified in Table 3 was added along with reagents for RT-PCR. The RT-PCR conditions were as described in Example 1 above. The primer rations used in this example were 1:1:1:1:10:40 (F_{out}:F_{in}:R_{in}:R_{out}:FSP:RSP).

The detection of the amplified products containing the target sequence was carried out as described for the direct detection methodology using Luminex beads as described in Example 1. Detection oligonucleotides for each agent listed in Table 3 were added to each detection reaction.

Table 6 shows the results of this experiment using the target enrichment and target amplification primers disclosed in Table 3. The rows represent the identity of the disease agent or secondary disease agent whose nucleic acid was used in the initial amplification reaction and the ratio of the primers used in the amplification step. The columns represent the detection oligonucleotides used in the detection step. In this example, sample number 1 is a RT-PCR Blank comprising a mixture of target enrichment and target amplification primers and detection oligonucleotides conjugated to Luminex beads was hybridized to the RT-PCR reaction that did not include template. The background signals are used to determine the cut-off values for a positive reaction. In a multiplexed system, such as the MAS utilizing TemPCR, each bead set is, in fact, a micro-system of its own. The final signal, as well as the background, is influenced by many factors including: the efficiency of the coupling reaction that links the capture oligonucleotides onto the bead sets; the efficiency of target amplification in the multiplexed

TemPCR reaction; and the efficiency of hybridization during detection. As a result, the cutoff value for each pathogen (represented by each bead set) must be decided individually. The background signals (determined by averaging the values obtained from the RT-PCR blank and the samples that were known not to contain the target agent) were determined and the standard deviation obtained. The standard deviation was multiplied by five (5) and this value added to the average background. Values higher than the average background are considered positive results, indicating the presence of a particular agent.

Samples 2-4 were adenoviruses subtype 4, 7, and 21, respectively. Samples 5-11 were *Chlamydia pneumoniae* (CPN), *Mycoplasma pneumoniae* (MPN), Influenza A (INFA), Influenza B (INFB), Parainfluenza type 1 (PIV-1), Parainfluenza type 3 (PIV-3), and Respiratory syncytial virus (RSV), respectively. Sample 12 was SARS-CoV. To increase detection sensitivity and minimize false negative detection caused by target mutations, we selected three different target sequences from the SARS-CoV genome for amplification and detection. Samples 13-16 were different enteroviruses, including Coxsackie virus A (CVA), Coxsackie virus B (CVB), Rhinovirus (RhV), and Echovirus (EV). For detection of adenovirus and enterovirus species, each of the specific detection nucleotides for adenovirus and enterovirus species were added in the detection step.

As can be seen in Table 6, the TemPCR method of the present disclosure provided specific amplification of the target sequences, which were specifically detected by the corresponding detection oligonucleotides. The high detection specificity was evident by the high signal to background ratios obtained from each bead set. This result indicates the target enrichment primers amplified the correct target nucleic acid sequence in the presence of multiple primer sets not specific for the target sequence and that the target amplification primers (FSP and RSP) function correctly to amplify the target sequence as described and the appropriate detection oligonucleotides are capable of hybridizing to the target sequence being detected.

Example 3

In Examples 1 and 2 above, only 1 nucleic acid sample was added to each reaction (albeit in the presence of target enrichment primers specific for multiple agents). Table 7 shows the specificity of the TemPCR amplification method when nucleic acid samples from multiple pathogens were included in a single sample for multiplex amplification and subsequent detection. In this example, target enrichment primers for each organisms listed in Table 3 were included in each sample, along with the target amplification primers and nucleic acid from the indicated pathogens. The rows in Table 7 indicate the detection oligonucleotide detected during the multiplex detection step (note that detection oligonucleotides specific for the target sequences for all the organisms listed in Table 3 were included in each detection reaction) while the rows in

Table 6 indicated the identity of the pathogen nucleic acid added to each sample. TemPCR amplification conditions and multiplex detection were carried out as described in Example 2 above.

Sample 1 is a negative control where no template nucleic acid was included in the RT-PCR reaction. Sample 2 included three pathogens, SARS, CPN, and INFA; Sample 3 included ADV-7, RSVB, and INFB; Sample 4 included PIV-3, CPN, and INFA; Sample 5 included ADV-21, RSVB, and INFB; Sample 6 included RhV and PIV1; Sample 7 included SARS and INFB; and Sample 8 included ADV-7 and PIV1. The cutoff values shown in Table 6 were used in this Example and values higher than the cutoff were highlighted in Table 7.

The results show that TemPCR correctly amplified the correct target sequence from each agent and that the amplified target sequences could be detected to provide an accurate and specific readout of the agents present in the sample. As one example, in row 2 nucleic acid from SARS, CPN and INFA was added to the amplification reaction. In the presence of the target enrichment primers specific for all the agents in Table 3, the TemPCR method using the primers disclosed correctly amplified the correct target sequences. No modification or optimization of the TemPCR amplification conditions was required to obtain correct multiplex amplification of the three agents. Importantly, the same set of TemPCR multiplex amplification conditions allowed the specific detection of a variety or of agents using a standardized amplification protocol. The TemPCR method described is highly specific and can detect specific pathogens in various combinations. No false positives or false negatives were observed.

Example 4

To validate the sensitivity of the TemPCR method, 1 ml serum samples were prepared with different amounts of viral or bacterial agents as listed in Table 8. For each agent, four concentrations were prepared: 10^4 pfu/ml, 10^3 pfu/ml, 10^2 pfu/ml, and 10^1 pfu/ml. To observe the assay repeatability, at each of the four concentrations, triplicate samples were prepared and analyzed. Certain samples, such as ADV4 and RhV, did not have high titer stocks available and the starting concentration was 10^3 pfu/ml. For SARS, the viral stock was limited, and therefore, only two samples were studied for each concentration (rather than three). A positive control sample at high concentration (not spiked into serum) was also included for each pathogen.

For each 1ml of the spiked sample serum, 200µl was used for nucleic acids isolation. Nucleic acid isolation was performed using the triazol method as described herein. At the end of the isolation procedure, the nucleic was eluted into 50µl RNAse free water. A volume of 5µl from this elution was used as template in a subsequent TemPCR reaction. Therefore, if the starting concentration was 10^4 pfu/ml, the TemPCR amplification reaction included only about 200 copies of pathogen genomes. Similarly, at 10^3 pfu/ml level, only 20 copies were included in the reaction

system and so on. Conditions for the TemPCR amplification reaction were as described above in Example 2 and multiplex detection carried out as described in Example 2 using the direct detection method.

Table 8 shows the results of the sensitivity study. A cutoff value was determined for each target specifically and the cutoff values were set to be the mean plus 5 times of the standard deviation. Values above the cutoff were considered positive and were highlighted in the Table 8. In general, the assay could detect between 20-200 copies of pathogens present in a serum sample. It should be noted that TemPCR conditions used in this Example did not incorporate the selective amplification step discussed herein. The use of the selective amplification step is expected to increase the sensitivity of the reaction significantly.

Example 5

Example 5 illustrates the results of an experiment utilizing an alternate embodiment of the TemPCR method. In this example, nucleic acid sequences from each disease agent and the secondary disease agents were obtained and isolated as described herein. The nucleic acid from each agent was amplified by the Tem-PCR method. In this embodiment, the amplification reaction conditions for TemPCR were altered to include a target enrichment step and a selective amplification step. The nucleic acid for each agent was placed in a separate reaction tube and the complete mix of primers specified in Table 3 was added along with reagents for RT-PCR. The primer rations used in this example were 1:1:1:1:10:40 (F_{out}:F_{in}:R_{in}:R_{out}:FSP:RSP).

The amplification conditions are given below. The reaction tubes were placed in a thermocycler programmed as follows: (i) reverse transcription- 30 minutes at 50^{0}C; (ii) initial PCR activation- 15 minutes at 95^{0}C; (iii) first amplification reaction comprising a first 3-step cycling (target enrichment)- 0.5 minutes at 94^{0}C, 1 minute at 52^{0}C and 1 minute at 72^{0}C for 15 complete cycles, and a second 2-step cycling (selective amplification)- 15 seconds at 94^{0}C, 1.5 minutes at 70^{0}C; for 6 complete cycles, preferably 4-8 complete cycles; (iv) second amplification reaction comprising a third 3-step cycling (target amplification)- 15 to 30 seconds at 94^{0}C, 15 to 30 seconds at 50-55^{0}C and 15 to 30 second at 72^{0}C; for at least 2 complete cycles, preferably for 10-40 complete cycles; and (v) final extension- 3 minutes at 72^{0}C. The detection of the amplified products containing the target sequence was carried out as described for the direct detection methodology using Luminex beads as described in Example 1 and illustrated in FIG. 2. Detection oligonucleotides for each agent listed in Table 3 were added to each detection reaction.

Table 9 shows the results of this experiment using the target enrichment and target amplification primers disclosed in Table 3. The rows represent the identity of the disease agent or secondary disease agent whose nucleic acid was used in the initial amplification reaction. As can be seen in Table 9, the TemPCR method utilizing the alternate amplification strategy provided

specific amplification of the target sequences, which were specifically detected by the corresponding detection oligonucleotides. The high detection specificity was evident by the high signal to background ratios obtained from each bead set. This result indicates the target enrichment primers amplified the correct target nucleic acid sequence in the presence of multiple primer sets not specific for the target sequence and that the target amplification primers (FSP and RSP) function correctly to amplify the target sequence as described and the appropriate detection oligonucleotides are capable of hybridizing to the target sequence being detected.

A subject of the present disclosure is also a kit comprising the components necessary for carrying out the method disclosed in all the embodiments illustrated. The kit may comprise one or more of the following: at least one set of primers to for the amplification of target sequences from a disease agent and secondary disease agent in sample from an individual suspecting of harboring the disease agent, reagents for the isolation of nucleic acid (RNA, DNA or both), reagents for the amplification of target nucleic acid from said sample (by PCR, RT-PCR or other techniques known in the art), microspheres, either with or without conjugated capturing reagents (in one embodiment, the cRTs), target sequence specific detection oligonucleotides, reagents required for positive/negative controls and the generation of first and second signals.

All references cited herein (including articles, patents and url addresses) are incorporated by reference to the extent allowed. The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior disclosure. The following claim is appended for the purpose of foreign priority only.

	DNA	RNA (+)	RNA (-)
F_{out}	1	1	4
R_{out}	1	4	1
F_{in}	1	1	1
R_{in}	1	1	1
FSP	8	8	8
RSP	32	32	32

Table 1- Exemplary ratios of nested primers and super primers for disease agents and secondary disease agents with the indicated genomes. The ratios of nested primers and super primers may be varied as discussed in the instant specification.

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Table 5. Description of the included pathogen targets and detectable strains.

No.	Pathogen	Name in Product	Tageted gene	Detectable types/strains
1	SARS-CoV	SARS1	5'end polyprotein	
		SARS2	Polymerase gene	
		SARS3	N gene	
2	Influenza A	INFA	NS gene	H1N1, H1N2, H2N2, H3N2, H3N8, H4N6, H4N8, H5N1, H5N2, H5N3, H6N1, H6N2, H6N4, H7N1, H7N2, H7N3, H7N7, H7N8, H9N2, H10N5, H11N1, H11N8, H11N9
3	Influenza B	INFB	NS gene	
4	Adenoviruses	ADV	Hexon gene	Type 3,4, 7, 14, and 21.
5	Parainfluenza1	PIV1	N gene	
6	Parainfluenza3	PIV3	N gene	
7	Respiratory syncytial virus	RSV	NS gene	RSVA and RSVB
8	M. pneumoniae	MPN	cytadhesin P1 gene	
9	C.pneumoniae	CPN	Uridine Kinase gene	
10	Enteroviruses	ENT	5' UTR	Rhinovirus 1a, 1b, 2, 9, 14, 15, 16, 39, 49, 50, 85, 89; Coxackie Viruse A: A21, A24; Coxsackie Viruse B: B4, B5; Echovirus 11, 20, and 25.

Table 2- Primer sequences and detection oligonucleotide sequences used in the multiplex amplification in one embodiment of the method disclosed.

GenBank ID	Target Name	Oligonucleotides sequences	Positions
AY278491	SARS		
	SARS1Fo	ACCGTAGACTCATCTCTATGATG	18121-18143
	SARS1Fi	CAGGCCACGTTTTGTCATGCGAAGCTATTCGTCACGTTCG	18201-18220
	SARS1Ro	TTGCATTAACTCTGGTGAATTCTG	18384-18361
	SARS1Ri	TTCTTTGCGTTATGTCTCTGCTGTAGAAAATCCTAGCTGG	18309-18290
	SARS1De	TAGAGGGCTGTCATGCAACT	18241-18260
	SARS2Fo	ATGCCTAACATGCTTAGGATAATG	15246-15269
	SARS2Fi	CAGGCCACGTTTTGTCATGCTTTCTACAGGTTAGCTAACGA	15323-15343
	SARS2Ro	TACATTGGCTGTAACAGCTTGAC	15482-15460
	SARS2Ri	TTCTTTGCGTTATGTCTCTGAGCATAAGCAGTTGTAGCATC	15440-15420
	SARS2De	GTGAGATGGTCATGTGTGGC	15361-15380
	SARS3Fo	ACAATGCTGCCACCGTGCTAC	28580-28600
	SARS3Fi	CAGGCCACGTTTTGTCATGCCCTCAAGGAACAACATTGCC	28606-28625
	SARS3Ro	TAGCGCGAGGGCAGTTTCAC	28785-28766
	SARS3Ri	TTCTTTGCGTTATGTCTCTGCCGCTAGCCATTCGAGCAGG	28760-28741
	SARS3De	ATCATCACGTAGTCGCGGTAA	28681-18700
M11486	RSVA		
	RSVAFo	ATTGGCATTAAGCCTACAAA	899-918
	RSVAFi	CAGGCCACGTTTTGTCATGCGGGCAAATACAAAGATGGCTC	1082-1102
	RSVARo	GACATAGCATATAACATACCTATT	1310-1287
	RSVARi	TTCTTTGCGTTATGTCTCTGGGAGTRTCAATAYTATCTCCTGT	1202-1180
	RSVADe	CACTCAACAAAGATCAACTT	1127-1146
D00736	RSVB		
	RSVBFo	ATTGGCATTAAGCCTACAAA	888-907
	RSVBFi	CAGGCCACGTTTTGTCATGCGGGCAAATACAAAGATGGCTC	1071-1091
	RSVBRo	GACATAGCATATAACATACCTATT	1299-1276
	RSVBRi	TTCTTTGCGTTATGTCTCTGGGAGTRTCAATAYTATCTCCTGT	1191-1169
	RSVBDe	CATTAAATAAGGATCAGCTG	1116-1135
AF457102	PIV-1		
	PIV1Fo	CACAATTGATATGAATTATTGG	13037-13058
	PIV1Fi	CAGGCCACGTTTTGTCATGCTGATGAAATAATTAGAGCAACTAG	13064-13087
	PIV1Ro	CTATTWATATCATCATCATTT	13186-13166

	PIV1Ri	TTCTTTGCGTTATGTCTCTGTGCTATCATTTCTTTAAGATTG	13160-13139
	PIV1De	CAGCTATGACTATTGCAGAC	13096-13115
Z11575	PIV-3		
	PIV3Fo	CACAATTGATATGAATTATTGG	12911-12932
	PIV3Fi	CAGGCCACGTTTTGTCATGCTACTGACATCATACATGCAATTTC	12938-12961
	PIV3Ro	CTATTWATATCATCATCATTT	13060-13040
	PIV3Ri	TTCTTTGCGTTATGTCTCTGTAACTATTATCTCTTTTAAATT	13035-13014
	PIV3De	CTGCAATTACAATAGCAGAT	12970-12989
NC_000912	M.Pneumoniae		
	MPMFo	ACCAGCATAAGAACCTCCTG	323867-323886
	MPMFi	CAGGCCACGTTTTGTCATGCTCAAGTCACGTACTCGCCATC	323891-232911
	MPMRo	TTAAACTGTTACTGTTGTGC	324086-324067
	MPMRi	TTCTTTGCGTTATGTCTCTGTTTGCGAGATCTCGAGGGGTC	324039-324019
	MPMDe	GCTGAATAAACCGGGTATTA	323961-323980
AE001618	C.Pneumoniae		
	CPMFo	GCCTGCCCTATGAAAACGATG	6780-6800
	CPMFi	CAGGCCACGTTTTGTCATGCCGTGATCCACACGAGTCATAC	6850-6870
	CPMRo	TAAAGCTGCTTCGGGAACGTG	6970-6950
	CPMRi	TTCTTTGCGTTATGTCTCTGTATCGGGGTTGTATTTCCTTC	6943-6923
	CPMDe	ATCGGAAGTCGCTCTATCTT	6885-6904
AY027864	Enterovirus		
	ENTVFo	CCTCCGGCCCCTGAATGCGG	1-20
	ENTVFi	CAGGCCACGTTTTGTCATGCCCTAACTGTGGAGCACATGCC	26-46
	ENTVRo	TGTCACCATAAGCAGCCAATG	152-132
	ENTVRi	TTCTTTGCGTTATGTCTCTGTAGTCGGTTCCGCTGCAGAG	100-81
	ENTVDe	CCAGAGGGTAGTGTGTCGTA	53-72
AJ344037	InfluenzaA		
	INFAFo	TGCAATTGGGGTCCTCATCGG	528-548
	INFAFi	CAGGCCACGTTTTGTCATGCTTGAATGGAATGATAACACAG	554-574
	INFARo	AAACGAGAAAGTTCTTATCTC	826-806
	INFARi	TTCTTTGCGTTATGTCTCTGGTTCTCGCCATTTTCCGTTTC	674-654
	INFADe	TCTACAGAGATTCGCTTGG	591-609
AF492482	InfluenzaB		
	INFBFo	TGAAGGGTTTGAGCCATACTG	291-311
	INFBFi	CAGGCCACGTTTTGTCATGCTACAATTGGACCGATTACCCT	346-366
	INFBRo	TGAGTGTTTACTTCCTCCTTTATC	497-474

	INFBRi	TTCTTTGCGTTATGTCTCTGGTTGTTCATGTCCCTTAATACT	456-435
	INFBDe	CCTTGATGACATAGAAGAAG	384-403
AF542122	Adenovirus		
	ADVFo	AACAGACCCAAYTACATTGG	910-929
	ADVFi	CAGGCCACGTTTTGTCATGCATGTACTACAACAGTACTGG	955-974
	ADVRo	TATGACAGTTCWGTGTTTCTGTC	1052-1033
	ADVRi	TTCTTTGCGTTATGTCTCTGGCAAGTCAACCACHGCATTC	1030-1011
AY008279	ADV21De	GAGTGCTGGCAGGTCAAGCA	1034-1053
AF542129	ADV3De	GAGTTTTGGCTGGCCAAGCA	1016-1035
AF542122	ADV4De	GGGTACTGGCCGGTCAGGCC	983-1002
AF515814	ADV7De	GAGTTTTGGCCGGCCAAGCA	1131-1150
AB018425	ADV14De	GGGTGCTGGCTGGCCAAGCA	717-736

Super primer sequences
FSP- CAGGCCACGTTTTGTCATGC
RSP- TTCTTTGCGTTATGTCTCTG

Table 3 Primer sequences and detection oligonucleotide sequences used in the multiplex amplification in one embodiment of the method disclosed.

SARS	Sequence 5' to 3'
SARS1Fo	ACCGTAGACTCATCTCTATGATG
SARS1Fi	CAGGCCACGTTTTGTCATGCGAAGCTATTCGTCACGTTCG
SARS1Ro	TTGCATTAACTCTGGTGAATTCTG
SARS1Ri	TTCTTTGCGTTATGTCTCTGCTGTAGAAAATCCTAGCTGG
SARS2Fo	ATGCCTAACATGCTTAGGATAATG
SARS2Fi	CAGGCCACGTTTTGTCATGCTTTCTACAGGTTAGCTAACGA
SARS2Ro	TACATTGGCTGTAACAGCTTGAC
SARS2Ri	TTCTTTGCGTTATGTCTCTGAGCATAAGCAGTTGTAGCATC
SARS4Fo	ACAATGCTGCCACCGTGCTAC
SARS4Fi	CAGGCCACGTTTTGTCATGCCCTCAAGGAACAACATTGCC
SARS4Ro	TAGCGCGAGGGCAGTTTCAC
SARS4Ri	TTCTTTGCGTTATGTCTCTGCCGCTAGCCATTCGAGCAGG
SARS1De	TAGAGGGCTGTCATGCAACT
SARS2De	GTGAGATGGTCATGTGTGGC
SARS4De	TCATCACGTAGTCGCGGTAA
RSV A	
RSVAFo	AAGAATTTGATAAGTACCAC
RSVAFi	CAGGCCACGTTTTGTCATGCACTCCCTTGGTTAGAGATGG
RSVARi	TTCTTTGCGTTATGTCTCTGCAATGCTACTTCATCATTGTC
RSVARo	TATGTATCACTGCCTTAGCC
RSVADe	GCAGCAATTCATTGAGTATG
RSV B	
RSVBFo	AATAAGAATTTGATAAGTGC
RSVBFi	CAGGCCACGTTTTGTCATGCACCTTTTCAATCAGAAATGG
RSVBRi	TTCTTTGCGTTATGTCTCTGCAATGCTACTTCGTCATTGTC
RSVBRo	TGCTTTGGCTAATGCATTGG
RSVBDe	GGTGCAATTCACTGAGCATG
PIV1	
PIV1Fo	AGTATCACTCCTTGCAATGG
PIV1Fi	CAGGCCACGTTTTGTCATGCATCTCACTACAAACGGTGTC
PIV1Ri	TTCTTTGCGTTATGTCTCTGTTTGACAATGAACCCATCTG
PIV1Ro	GTTCTTTCATACTCCATGTC
PIV1De	GCTGATGTCAAGTATGTGAT
PIV3	
PIV3Fo	TCAATGGCTTATGCCAATCC
PIV3Fi	CAGGCCACGTTTTGTCATGCACAACAAATGGAAGTAATGC
PIV3Ri	TTCTTTGCGTTATGTCTCTGCTCGTCTTAACCACAAATCC
PIV3Ro	CAGGTCACTTCCAAATATCC
PIV3De	CTAAAACGGCAAAAGTATGG
InfA	
INFAFo	TGCAATTGGGGTCCTCATCGG
INFAFi	CAGGCCACGTTTTGTCATGCTTGAATGGAATGATAACACAG
INFARo	AAACGAGAAAGTTCTTATCTC

INFARi	TTCTTTGCGTTATGTCTCTGGTTCTCGCCATTTTCCGTTTC
INFADeC	TCTACAGAGATTCGCTTGG
INFB	
INFBFo	AGTCTTATCCCAATTTGGTC
INFBFi	CAGGCCACGTTTTGTCATGCAGAGCACCGATTATCACCAG
INFBRi	TTCTTTGCGTTATGTCTCTGCATGTCAGCTATTATGGAGC
INFBRo	AAGCACTGCCTGCTGTACAC
INFBDe	TTCCACAAAACAGTAATAGC
MPN	
MPNFo	ATCACCTTTAACCCCTTTGG
MPNFi	CAGGCCACGTTTTGTCATGCCGGCTTTGGTTTGAGTGGG
MPNRi	TTCTTTGCGTTATGTCTCTGCGCGGCACGAGTAAAACGGC
MPNRo	TGCAACTGCTCATAGTACAC
MPNDe	TGCACCCCAACAGTGAAACG
CPN	
CPN 5 Fo	GAAATTTATAGAGCCGACTCG
CPN 5 Fi	CAGGCCACGTTTTGTCATGCGCTGATATCATTGTACATGG
CPN 5 Ro	GTTGACCATATAATACGTCTC
CPN 5 Ri	TTCTTTGCGTTATGTCTCTGGCTTTCCAGGGCATTCTC
CPN5 De	ACCGACAAAACGTAGTAACA
ENTV	
CVA2 Fo	CAAGGTGTGAAGAGCCTATTG
CVBEV Fo	CATGGTGCGAAGAGTCTATTG
RhV2 Fo	GTGAAGAGCC(GC)CGTGTGCTC
ENT3 Fi	TTCTTTGCGTTATGTCTCTGAGTCCTCCGGCCCCTGAATG
ENT3 Ri	CAGGCCACGTTTTGTCATGCAAACACGGACACCCAAAGTAG
CVEV Ro	ATTGTCACCATAAGCAGCC
RhV2 Ro	TATATATTGTCACCATAAGC
CVEV De	GTTAGGATTAGCCGCATTCA
RhV2 De	GTTGGTCCCATCCCGCAATT
ADV	
ADV3-3Fi	CAGGCCACGTTTTGTCATGCCCCATGGATGAGCCCACCC
ADV3-3Ri	TTCTTTGCGTTATGTCTCTGGCTGGTGCACTCTGACCACG
ADV4-3Ri	TTCTTTGCGTTATGTCTCTGGCTGGTGCACTCGGACGACG
ADV14-3Ri	TTCTTTGCGTTATGTCTCTGGCTGATGCACTCTGACCACG
ADV3-3Fo	AGCAACTTCATGTCYATGGG
ADV3-3Ro	GTGCGCAGGTAGACGGCCTC
ADV14-3Ro	GTACGCAGGTAGACTGTCTC
ADV3-3De	GCTTTATCTTCTTTTCGAAG
ADV4-3De	TCTCTATGTTGTCTTCGAAG
ADV14-3De	GCTTTATCTTCTCTTCGAAG
Superprimers	
FSP	CAGGCCACGTTTTGTCATGC
RSP	TTCTTTGCGTTATGTCTCTG

Table 4

	Sample	RSVB	SARS1	SARS2	SARS3	PIV-1	MPN	CPN	ENT	INFA	INFB	ADV3	ADV7	ADV14	ADV21
1	LUM Blank	20	12	16	24	21	16	19	17	20	22	25	25	25	32
2	Blank 4:1:1:1:8:16	16	7	8	13	15	16	12	16	12	19	10	13	11	14
3	Blank 4:1:1:1:8:24	16	25	10	10	15	15	16	12	22	14	16	15	14	18
4	Blank 4:1:1:1:8:32	16	26	21	13	4	10	13	24	16	18	14	17	14	24
5	RSVB 4:1:1:1:8:16	1102	14	20	13	14	7	10	16	14	17	13	17	17	19
6	RSVB 4:1:1:1:8:24	1377	12	8	10	14	7	14	12	19	24	17	11	11	28
7	RSVB 4:1:1:1:8:32	1496	26	10	10	11	13	10	13	15	14	9	12	16	19
8	PIV1 4:1:1:1:8:16	19	22	10	11	311	18	12	19	10	15	10	18	26	18
9	PIV1 4:1:1:1:8:24	9	19	15	12	298	12	18	19	13	12	13	13	12	20
10	PIV1 4:1:1:1:8:32	13	38	17	9	514	20	22	29	15	23	11	13	16	16
11	INFA 4:1:1:1:8:16	10	31	15	17	8	13	14	119	260	19	18	11	22	30
12	INFA 4:1:1:1:8:24	26	46	23	8	9	10	13	186	454	15	9	9	15	23
13	INFA 4:1:1:1:8:32	21	91	28	17	7	10	16	323	630	42	10	16	22	29
14	INFB 4:1:1:1:8:16	10	22	18	27	14	9	6	13	16	677	12	21	16	23
15	INFB 4:1:1:1:8:24	6	14	12	37	6	11	7	16	14	1162	10	15	12	23
16	INFB 4:1:1:1:8:32	5	29	21	51	11	9	13	23	12	1480	11	14	13	20
17	ADV21 4:1:1:1:8:16	8	10	19	9	13	19	13	21	13	17	11	13	12	286
18	ADV21 4:1:1:1:8:24	9	18	12	8	12	10	15	21	13	17	13	15	14	452
19	ADV21 4:1:1:1:8:32	12	43	17	8	11	6	11	25	13	19	11	10	10	543
20	MPN 4:1:1:1:8:16	16	16	8	19	4	330	16	16	20	16	19	17	13	25
21	MPN 4:1:1:1:8:24	15	29	27	15	3	1182	21	19	21	17	18	20	16	23
22	MPN 4:1:1:1:8:32	10	30	21	16	14	1317	12	27	13	25	15	19	20	23
23	CPN 4:1:1:1:8:16	14	22	12	12	7	19	1109	19	17	25	13	13	13	19
24	CPN 4:1:1:1:8:24	23	27	15	12	7	10	1342	18	16	21	19	13	17	23
25	CPN 4:1:1:1:8:32	13	43	30	13	8	20	1828	18	13	30	11	15	17	17
26	SARS 4:1:1:1:8:16	11	857	768	227	12	17	11	14	20	20	14	17	21	20
27	SARS 4:1:1:1:8:24	11	1251	1097	376	15	17	11	18	17	17	15	19	15	24
28	SARS 4:1:1:1:8:32	7	1638	1528	693	9	13	6	20	16	19	14	13	14	17

RSV= respiratory syncytial virus; PIV= parainfluenza virus; INF= influenza virus; ADV+ adenovirus; MPN= *Mycoplasma pneumonia*; CPN= *Chlamydia pneumonia*; SARS= severe acute respiratory syndrome virus

Table 6

RSV= respiratory syncytial virus; PIV= parainfluenza virus; INF= influenza virus; ADV+ adenovirus; MPN= *Mycoplasma pneumonia*; CPN= *Chlamydia pneumonia*; SARS= severe acute respiratory syndrome virus

Sample/Target		ADV	CPN	MPN	INFA	INFB	PIV1	PIV3	RSV	SARS1	SARS2	SARS4	ENT
1	RT-PCR Blank	13	8	12	15	10	10	27	4	6	10	8	9
2	ADV-4	1134	17	7	20	7	11	20	5	4	6	4	4
3	ADV-7	530	8	10	8	7	10	23	8	3	10	4	13
4	ADV-21	968	17	7	14	13	15	29	6	9	11	9	14
5	CPN	21	661	13	19	24	27	66	6	5	9	12	14
6	MPN	11	33	1696	25	21	21	77	17	16	7	12	12
7	INFA	25	32	16	809	73	22	105	17	10	8	13	12
8	INFB	19	41	10	21	2061	27	89	12	10	11	18	13
9	PIV-1	24	30	9	12	24	1705	65	11	10	10	12	13
10	PIV-3	26	41	14	25	35	40	1802	19	12	15	14	20
11	RSV	17	34	9	21	18	23	76	1782	12	22	13	8
12	SARS	26	36	16	27	37	21	74	16	2374	1934	437	1
13	CVA	15	43	17	25	31	27	80	17	19	10	17	2833
14	CVB	58	39	17	30	38	28	85	15	14	16	18	3254
15	RhV	12	19	7	20	8	13	34	12	9	2	8	1329
16	EV	6	14	14	17	9	12	31	8	24	10	6	159
Average Bkg		**21.0**	**27.4**	**11.7**	**19.7**	**23.6**	**20.4**	**58.6**	**11.4**	**10.7**	**10.4**	**11.1**	**10.9**
Standard deviation		**12.8**	**12.3**	**3.8**	**6.0**	**17.6**	**8.6**	**28.4**	**5.2**	**5.8**	**4.7**	**4.6**	**4.9**
Cutoff		**85.1**	**88.8**	**30.6**	**49.7**	**111.7**	**63.4**	**200.4**	**37.2**	**39.5**	**33.8**	**34.0**	**35.5**
Signal/Background		**41.9**	**24.2**	**144.5**	**41.0**	**87.3**	**83.6**	**30.8**	**155.9**	**221.2**	**186.6**	**39.3**	**173.5**

Table 7

RSV= respiratory syncytial virus; PIV= parainfluenza virus; INF= influenza virus; ADV+ adenovirus; MPN= *Mycoplasma pneumonia*; CPN= *Chlamydia pneumonia*; SARS= severe acute respiratory syndrome virus

Sample/Target		ADV	CPN	MPN	INFA	INFB	PIV1	PIV3	RSV	SARS1	SARS2	SARS4	ENT
1	RT-PCR Blank	35	25	18	24	32	18	45	12	9	7	10	12
2	SARS/CPN/INFA	8	93	10	630	36	13	70	10	746	823	104	11
3	ADV-7/RSVB/INFB	303	23	8	29	1482	15	61	1823	11	13	12	11
4	PIV-3/CPN/INFA	16	152	8	664	41	17	1156	13	7	11	9	9
5	ADV-21/RSVB/INFB	983	23	10	28	1665	19	53	1845	11	9	3	11
6	RhV/PIV-1	26	21	7	31	32	1712	51	14	6	7	11	958
7	SARS/INFB	18	15	9	19	1458	20	41	10	1799	1560	316	15
8	ADV-7/PIV-1	593	18	12	22	27	1611	49	15	13	16	14	16
Cutoff values		**85.1**	**88.8**	**30.6**	**49.7**	**111.7**	**63.4**	**200.4**	**37.2**	**39.5**	**33.8**	**34.0**	**35.5**

Table 8

Table 4. The sensitivity of the assay system

Sample	Target	ADV	CPN	MPN	INFA	INFB	PIV1	PIV3	RSV	SARS1	SARS2	SARS3	EN1
1	RT-PCR Blank	8	35	7	21	37	16	85	16	13	5	14	9
2	Serum only	12	40	11	29	37	10	71	9	8	6	9	9
3	Serum only	13	32	7	24	34	13	76	9	6	4	11	7
4	Serum only	11	31	6	27	28	19	85	8	10	5	11	10
5	ADV4 10e3 ctrl	976	38	15	32	22	20	76	6	7	9	19	18
6	ADV4 10e3 #1	486	45	12	36	20	18	82	10	9	14	15	24
7	ADV4 10e3 #2	112	42	6	31	24	23	93	13	23	20	16	19
8	ADV4 10e3 #3	105	40	20	27	20	26	81	16	9	10	6	17
9	ADV4 10e2 #1	122	41	20	35	38	27	91	18	14	12	16	23
10	ADV4 10e2 #2	162	42	16	28	23	27	54	23	10	15	16	18
11	ADV4 10e2 #3	35	42	12	38	23	22	67	8	15	10	7	2
12	ADV4 10e1 #1	31	39	15	32	29	26	82	8	15	16	13	22
13	ADV4 10e1 #2	25	33	23	38	16	26	63	20	18	13	24	14
14	ADV4 10e1 #3	25	28	22	25	10	28	59	21	21	18	10	11
28	CPN 10e4 ctrl	23	362	17	34	24	30	66	18	11	14	16	45
29	CPN 10e4 #1	10	256	12	33	24	26	73	18	14	15	13	9
30	CPN 10e4 #2	18	111	9	34	27	24	98	7	13	13	22	13
31	CPN 10e4 #3	16	239	8	24	19	21	75	17	19	9	17	32
32	CPN 10e3 #1	16	103	13	31	26	26	88	11	17	8	9	10
33	CPN 10e3 #2	14	43	12	41	23	23	91	14	10	12	13	9
34	CPN 10e3 #3	15	85	12	35	15	27	79	14	10	17	17	7
35	CPN 10e2 #1	11	33	9	28	19	18	56	15	10	6	7	19
36	CPN 10e2 #2	6	35	8	34	23	26	53	13	11	10	6	12
37	CPN 10e2 #3	19	42	11	24	22	20	63	9	15	10	11	19
38	CPN 10e1 #1	11	42	10	31	13	17	65	4	10	7	13	17
39	CPN 10e1 #2	22	32	17	25	20	24	47	14	15	15	13	4
40	CPN 10e1 #3	13	32	13	29	17	25	41	12	8	8	13	17
41	MPN 104 ctrl	12	34	578	29	32	19	75	11	8	12	6	11
42	MPN 104 #1	13	33	269	24	39	12	76	12	6	10	9	6
43	MPN 104 #2	9	28	354	21	17	15	57	12	8	4	12	9
44	MPN 104 #3	12	30	428	23	18	15	55	8	4	4	15	12
45	MPN 103 #1	14	34	109	22	21	12	88	11	13	12	7	7
46	MPN 103 #2	11	37	23	28	19	17	95	9	13	10	14	6
47	MPN 103 #3	8	37	82	30	22	12	72	7	7	7	8	9
48	MPN 102 #1	6	54	9	36	21	21	124	12	10	11	7	17
49	MPN 102 #2	12	42	18	28	22	7	96	9	1	13	13	10
50	MPN 102 #3	13	39	19	29	18	10	106	17	7	4	9	11
51	MPN 101 #1	8	38	11	26	21	16	101	16	4	3	14	0
52	MPN 101 #2	11	38	9	32	26	17	102	10	5	12	11	10
53	MPN 101 #3	13	34	15	31	19	11	91	14	12	6	11	10
54	INFA 10e4 ctrl	16	35	16	840	36	14	92	13	16	12	13	17
55	INFA 10e4 #1	20	39	18	512	38	21	83	17	9	7	9	13
56	INFA 10e4 #2	17	33	14	339	43	15	92	14	13	11	10	17
57	INFA 10e4 #3	11	45	19	478	42	23	101	8	11	14	17	21
58	INFA 10e3 #1	14	46	10	103	20	15	67	16	5	8	15	8
59	INFA 10e3 #2	20	41	11	113	26	11	93	18	9	15	6	13
60	INFA 10e3 #3	11	45	11	206	25	14	76	17	7	13	11	17
61	INFA 10e2 #1	12	26	7	49	15	17	47	12	5	8	11	21
62	INFA 10e2 #2	14	21	6	97	24	22	47	18	13	5	18	16
63	INFA 10e2 #3	15	32	7	90	18	18	38	20	4	6	15	12
64	INFA 10e1 #1	12	27	14	36	3	19	48	14	12	16	6	13
65	INFA 10e1 #2	16	26	12	31	14	20	41	14	12	7	9	9
66	INFA 10e1 #3	23	37	12	28	23	13	40	12	16	11	17	19
67	INFB 10e4 ctrl	17	24	11	55	349	14	47	10	10	13	13	7
68	INFB 10e4 #1	11	32	10	36	174	21	48	15	8	12	14	19
69	INFB 10e4 #2	12	30	5	32	266	19	56	10	11	9	8	4
70	INFB 10e4 #3	13	28	13	26	277	20	53	12	15	15	13	12
71	INFB 10e3 #1	13	31	15	20	20	16	61	14	12	15	10	10
72	INFB 10e3 #2	10	30	11	22	20	19	43	13	12	6	12	6
73	INFB 10e3 #3	16	35	14	29	21	22	53	9	2	15	16	13
74	INFB 10e2 #1	12	29	11	24	22	17	50	16	12	6	13	18
75	INFB 10e2 #2	15	28	8	19	22	19	65	15	11	16	17	13
76	INFB 10e2 #3	20	38	18	31	23	17	67	8	12	5	11	16
77	INFB 10e1 #1	11	35	7	30	44	12	76	8	18	5	9	14
78	INFB 10e1 #2	19	44	13	28	42	14	96	13	2	8	12	7
79	INFB 10e1 #3	18	30	4	28	25	17	69	12	6	3	16	14
80	PIV-1 10e4 ctrl	12	69	16	23	33	980	94	15	7	12	20	15
81	PIV-1 10e4 #1	16	34	8	20	18	461	47	8	17	5	18	9
82	PIV-1 10e4 #2	17	42	11	21	22	355	40	8	12	9	15	6
83	PIV-1 10e4 #3	14	32	10	14	16	630	38	10	9	17	12	6
84	PIV-1 10e3 #1	10	31	8	13	13	145	39	9	10	10	13	4
85	PIV-1 10e3 #2	22	46	6	13	13	149	54	13	19	11	15	16
86	PIV-1 10e3 #3	12	35	12	20	10	98	52	12	15	10	15	12
87	PIV-1 10e2 #1	15	40	6	16	4	26	37	6	7	12	13	9
88	PIV-1 10e2 #2	17	44	11	20	11	27	57	9	13	9	9	10
89	PIV-1 10e2 #3	22	38	13	18	10	35	57	12	9	9	16	9
90	PIV-1 10e1 #1	14	41	16	22	15	19	56	16	15	3	10	9
91	PIV-1 10e1 #2	9	46	14	24	14	10	45	10	11	13	13	13
92	PIV-1 10e1 #3	9	41	5	11	18	22	53	15	10	15	12	11

Table 4 continued

Sample/Target		ADV	CPN	MPN	INFA	INFB	PIV1	PIV3	RSV	SARS1	SARS2	SARS3	ENT
93	PIV-3 10e4 ctrl	12	54	16	30	33	25	1241	16	11	15	13	15
94	PIV-3 10e4 #1	12	58	5	29	18	31	290	21	8	7	11	5
95	PIV-3 10e4 #2	22	62	20	42	22	33	272	14	20	15	20	25
96	PIV-3 10e4 #3	13	49	8	30	16	21	229	13	7	0	10	21
97	PIV-3 10e3 #1	17	67	9	34	13	14	214	15	8	18	12	17
98	PIV-3 10e3 #2	18	70	15	39	13	21	301	11	9	7	11	11
99	PIV-3 10e3 #3	12	64	21	40	10	32	265	15	11	18	15	23
100	PIV-3 10e2 #1	17	67	16	23	4	29	171	15	20	10	17	7
101	PIV-3 10e2 #2	21	67	8	31	11	21	189	13	21	6	17	6
102	PIV-3 10e2 #3	16	67	9	36	10	36	181	19	10	13	9	15
103	PIV-3 10e1 #1	12	76	14	34	15	30	190	8	12	10	9	11
104	PIV-3 10e1 #2	13	70	14	32	14	35	215	21	4	8	11	15
105	PIV-3 10e1 #3	19	26	13	32	18	33	36	13	11	10	19	12
106	RSVB 10e4 ctrl	19	47	7	16	25	16	82	1232	11	9	15	18
107	RSVB 10e4 #1	11	47	16	32	31	18	93	1154	7	9	11	24
108	RSVB 10e4 #2	13	48	14	25	33	22	91	905	10	9	12	16
109	RSVB 10e4 #3	13	39	14	25	21	21	76	948	5	8	9	15
110	RSVB 10e3 #1	6	38	17	28	14	26	73	1149	19	17	15	20
111	RSVB 10e3 #2	16	42	6	21	21	20	76	1217	11	15	19	16
112	RSVB 10e3 #3	14	42	8	29	32	28	84	1198	6	9	12	24
113	RSVB 10e2 #1	11	44	16	33	29	17	100	40	14	16	7	21
114	RSVB 10e2 #2	6	39	16	23	21	21	88	23	15	9	11	14
115	RSVB 10e2 #3	20	41	12	20	36	22	90	29	9	17	13	25
116	RSVB 10e1 #1	23	49	10	29	30	18	95	12	16	11	7	17
117	RSVB 10e1 #2	15	44	15	27	35	17	94	11	11	7	5	18
118	RSVB 10e1 #3	13	54	20	35	33	24	91	23	10	16	9	16
119	SARS 10e4 ctrl	34	34	13	14	33	27	34	15	2121	1686	344	19
120	SARS 10e4 #1	33	29	12	18	36	19	30	17	2066	1742	337	14
121	SARS 10e4 #2	36	27	15	21	32	29	31	18	1514	1438	274	18
122	SARS 10e3 #1	44	37	16	17	31	33	31	20	1245	1316	252	13
123	SARS 10e3 #2	27	28	14	14	24	25	33	17	930	1069	180	11
124	SARS 10e2 #1	53	27	17	13	20	28	30	17	479	664	119	12
125	SARS 10e2 #2	42	24	13	21	17	27	29	15	196	381	53	9
126	SARS 10e1 #1	46	22	8	17	17	24	36	15	88	168	37	15
127	SARS 10e1 #2	10	16	14	23	10	17	28	10	6	4	5	18
128	RhV 10e4 ctrl	7	31	10	22	25	19	76	10	10	9	9	838
129	RhV 10e3 #1	11	29	11	32	25	16	83	11	9	10	14	737
130	RhV 10e3 #2	11	29	3	27	18	10	69	11	10	4	8	835
131	RhV 10e3 #3	13	32	9	25	16	17	66	15	6	7	9	775
132	RhV 10e2 #1	9	35	12	15	12	18	56	10	6	7	9	116
133	RhV 10e2 #2	11	33	11	25	12	14	58	7	14	8	6	133
134	RhV 10e2 #3	14	23	12	16	14	23	60	11	11	9	4	104
135	RhV 10e1 #1	14	31	22	20	21	22	58	12	13	11	8	23
136	RhV 10e1 #2	5	36	17	27	22	22	69	11	5	8	9	22
137	RhV 10e1 #3	14	28	11	24	17	23	59	13	8	10	15	16
138	CVA 10e4 ctrl	16	10	6	8	12	16	15	4	12	5	11	1837
139	CVA 10e4 #1	8	22	10	17	12	10	20	10	5	5	9	1285
140	CVA 10e4 #2	9	22	6	16	7	16	27	7	10	5	10	1288
141	CVA 10e4 #3	4	20	12	30	9	12	20	5	7	7	9	966
142	CVA 10e3 #1	7	30	12	23	30	15	73	12	11	10	10	146
143	CVA 10e3 #2	1	31	9	19	25	12	61	14	0	5	6	149
144	CVA 10e3 #3	10	40	9	16	22	10	65	5	4	6	3	194
145	CVA 10e2 #1	4	16	8	14	9	16	32	14	5	3	5	18
146	CVA 10e2 #2	10	26	5	21	11	9	38	7	7	11	5	86
147	CVA 10e2 #3	14	21	6	16	17	11	35	14	2	8	11	16
148	CVA 10e1 #1	9	21	5	21	12	16	31	3	2	5	9	9
149	CVA 10e1 #2	9	33	13	15	11	18	43	6	11	10	6	9
150	CVA 10e1 #3	11	30	6	18	26	10	58	10	8	9	5	20
151	CVB 10e4 ctrl	9	15	6	10	8	13	18	11	12	10	14	2117
152	CVB 10e4 #1	8	30	4	25	26	12	41	2	2	11	12	515
153	CVB 10e4 #2	9	26	8	13	21	16	33	6	6	6	5	395
154	CVB 10e4 #3	13	30	9	12	10	13	38	8	9	5	8	144
155	CVB 10e3 #1	6	23	15	24	21	15	37	9	8	8	11	106
156	CVB 10e3 #2	10	23	6	20	12	8	23	11	2	0	6	14
157	CVB 10e3 #3	12	22	7	28	13	7	34	16	6	4	10	84
158	CVB 10e2 #1	9	23	2	16	10	6	29	8	5	6	10	20
159	CVB 10e2 #2	11	27	5	17	15	23	26	17	12	12	9	20
160	CVB 10e2 #3	4	21	13	16	9	13	28	12	12	6	12	8
161	CVB 10e1 #1	5	24	5	8	12	16	18	7	9	8	9	2
162	CVB 10e1 #2	10	16	14	23	10	17	28	10	6	4	5	7
163	CVB 10e1 #3	15	18	10	21	15	17	29	8	2	11	10	8
mean of background		14.2	36.2	11.3	24.8	20.5	19.1	60.9	12.2	9.9	9.5	11.4	13.7
Standard deviation		7.9	12.7	4.4	7.5	9.0	6.3	23.9	4.2	4.5	4.1	4.0	6.4
Cutoff		53.5	99.5	33.6	62.2	65.7	50.9	180.2	32.9	32.4	30.0	31.5	45.7

Table 9

Sample	SARS1	SARS2	SARS4	RSVA	RSVB	PIV-1	PIV-3	INFA	INFB	MPN	CPN	CVEV	ADV 3-7-21	ADV 4	ADV 14
SARS	688	614	183	6	11	12	0	22	10	0	7	3	12	4	9
RSVA	25	39	17	3388	24	33	11	12	18	32	21	34	71	24	15
RSVB	13	19	27	20	2538	18	8	29	25	27	25	26	43	22	6
PIV-1	33	7	33	19	14	2008	19	23	13	19	28	23	13	19	5
PIV-3	15	21	29	20	12	25	1189	14	22	22	29	24	49	15	22
InfA	29	29	29	8	19	39	29	759	19	27	28	19	34	9	19
InfB	17	22	15	19	11	35	11	34	795	11	32	14	20	19	22
MPN	7	25	31	20	31	22	18	29	27	2304	37	21	41	27	9
CPN	22	11	18	18	23	20	23	18	14	21	1626	34	32	22	3
Sample Skipped	9	28	16	31	17	30	17	16	14	11	28	25	26	8	11
CVA-21 & EV-11	25	22	24	12	24	28	24	25	35	18	16	1376	32	5	15
CVB-4	25	17	22	19	25	21	23	30	28	25	18	1276	28	24	12
RhV	32	23	29	18	14	35	28	18	21	32	27	18	24	36	20
ADV 3	19	36	21	39	23	23	24	35	15	16	26	19	2458	15	88
ADV 4	20	12	18	24	18	26	7	20	29	29	37	28	520	1242	26
ADV 7	31	11	27	31	21	22	29	26	39	28	20	29	1758	21	28
ADV 14	36	20	16	23	15	18	21	24	13	20	17	19	654	9	1697
ADV 21	17	20	26	19	18	29	19	9	9	21	23	14	2235	15	90
RT-PCR Blank 1	22	6	14	20	18	24	15	31	14	26	18	4	28	9	10
RT-PCR Blank 2	15	13	30	6	17	7	27	23	9	6	24	24	13	2	15